Investigating the induction of autophagy by different *Mycobacterium tuberculosis* strains: Do strain-specific differences exist?

By
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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature……Date……March 2017……..

ABSTRACT

The pathogenicity of *Mycobacterium tuberculosis* (*M.tb*) is determined by its ability to survive within host macrophages. The mammalian autophagy pathway is now recognised as a major contributor to disease pathogenesis. Autophagy, a destructive catabolic process, plays a significant role in the destruction of intracellular pathogens. Clearer understanding of the natural range of autophagic responses elicited by different mycobacteria is required.

Autophagy induction has been shown to differ in magnitude depending on the mycobacterial species. However, no study has investigated the specific autophagic capacities of different *M.tb* strains. We aim to investigate the host autophagic response to different *M.tb* strains (and clades within strains) responsible for the tuberculosis epidemic in South Africa.

THP-1 cells were infected with seven different *M.tb* clinical isolates, representing six different lineages and the lab strain H37Rv. After RNA extraction, gene expression analysis of 84 autophagy-related genes was performed using the RT^2 Profiler™ autophagy array.

Our results revealed that all seven strains influenced the autophagy pathway in various ways and different magnitudes. Infection with the LAM 1 and CAS/Kili strains resulted in significant down-regulation of interferon gamma (*IFNG*) gene expression compared to the other stains. Since *IFNG* is a potent inducer of autophagy, we conclude that these two strains are weak inducers of autophagy.

The autophagosome formation is regulated through the *ATG1-10, ATG12-14*, *ATG16-18, ATG29* and *ATG31* genes. The LAM 1, Atypical Beijing, H37Rv, CAS/Kili and LCC strains have the ability to inhibit autophagosome formation, whereas Typical Beijing and Haarlem 3 induces the formation of autophagosomes.

Differential expression of genes involved with fusion of autophagosomes to lysosomes, *LAMP1*, *DRAM*, *GABARAP* and *NPC1*, showed that all the investigated strains impaired autophagolysosomal fusion. This result is not unexpected, since it is known that *M.tb* is able to block autophagolysosomal fusion. Furthermore, the LCC and LAM 1 impede the formation of the autophagic vacuole, while LAM 1 also influences protein transport, protein targeting to membrane/vacuole and protease activity.

The top 30 differentially expressed genes were subsequently investigated as potential TB susceptibility genes by analysing single nucleotide polymorphism(s) (SNPs) data generated using the Illumina Multi-ethnic Genotyping Array (MEGA) in a cohort of South African Coloured TB patients and control individuals. After conducting a case-control association study, none of the variants in the top 30 differentially expressed autophagy associated genes were associated with TB susceptibility following Bonferonni correction for multiple testing. This study improves our understanding of how *M.tb* manages to overcome the host immune system and points to genes exploited by specific strains during this process.

OPSOMMING

Die patogenesiteit van *Mycobacterium tuberculosis* (*M.tb*) word bepaal deur die vermoë om te oorleef binne die gasheer-makrofage. Die soogdier-gasheer se outofagie-meganisme word nou erken as 'n belangrike bydraende faktor wat die siekte se patologiese uitkoms bepaal. Outofagie, 'n vernietigende kataboliese proses, speel 'n belangrike rol in die vernietiging van intrasellulêre patogene. 'n Beter begrip van die natuurlike verskeidenheid van outofagie-reaksies, wat deur verskillende mikobakterieë ontlok word, is nodig.

Dit is reeds bewys dat die induksie van outofagie verskil na gelang van die mikobakteriële spesies. Daar is egter geen vorige studies wat die spesifieke outofagie-vermoëns van verskillende *M.tb*-stamme ondersoek het nie. In die huidige studie was ons doel om die gasheer se outofagie-reaksie op verskillende *M.tb*-stamme (en families binne stamme), wat verantwoordelik is vir die tuberkulose-epidemie in Suid-Afrika, te ondersoek.

THP-1-selle is met sewe verskillende *M.tb*-kliniese isolate geïnfekteer, wat ses verskillende families en die laboratorium-stam H37Rv insluit. Na RNA-isolasie, is die uitdrukking van 84 outofagie-verwante gene met behulp van die "RT^2 Profiler™ autophagy array" ontleed.

Ons resultate het aangetoon dat al sewe stamme die outofagie-pad op verskeie wyses en met verskillende grade beïnvloed. Dit het getoon dat infeksie met die stamme LAM 1 en CAS/Kili die vermoë het om betekenisvolle afregulering van die interferon gamma (*IFNG*)-geen in vergelyking met die ander stamme teweeg te bring. Aangesien *IFNG* 'n kragtige induseerder van outofagie is, kan ons aflei dat hierdie twee stamme swak induseerders van outofagie is.

Die vorming van outofagosome word deur die *ATG1-10-*, *ATG12-14-*, *ATG16-18-*, *ATG29-* en *ATG31*-gene gereguleer. Die LAM 1-, Atipiese Beijing-, H37Rv-, CAS/Kili- en LCC-stamme het die vermoë om outofagosome se vorming te inhibeer, terwyl Tipiese Beijing- en Haarlem 3-stamme die vorming van outofagosome induseer.

Die differensiële uitdrukking van gene betrokke by die samesmelting van outofagosome en lisosome, naamlik *LAMP1, DRAM, GABARAP* en *NPC1*, het aangedui dat al die stamme, wat ondersoek is, outofagolisosomale fusie kan ontwrig. Hierdie resultaat was te wagte, aangesien dit bekend is dat *M.tb* in staat is om outofagolisosomale vorming te blokkeer. In aansluiting hierby het LCC en LAM 1 verder die vermoë om die vorming van die outofagie-vakuool te blokkeer, terwyl LAM 1 ook proteïen-transport, proteïen-teikening van die membraan/vakuool en protease-aktiwiteit beïnvloed.

Die eerste 30 gene, wat differensieel uitgedruk word, is daarna ondersoek as moontlike gene, wat TB-vatbaarheid verhoog deur die ontleding van data aangaande enkel-nukleotied-polimorfisme(s) (SNPs), wat gegenereer is deur gebruik van die “Illumina Multi-ethnic Genotyping Array (MEGA)” op die DNS van 'n studie-groep Suid-Afrikaanse Kleurling TB-pasiënte en gesonde individue. Na die uitvoering van ‘n gevalle-kontrole – assosiasie-studie is geeneen van die eerste 30 diferensieël-uitgedrukte gene geïdentifiseer wat kandidaat-gene vir TB-vatbaarheid, ná Bonferroni-korreksie vir meervoudige toetse, was nie. Hierdie studie sal bydrae om ons begrip van hoe *M.tb* daarin slaag om die gasheer se immuunstelsel te oorkom, te verbreed. Dit dui ook aan watter gene deur spesifieke stamme gedurende hierdie proses uitgebuit word.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
µg	Microgram
µL	Microliter
AFB	Acid-Fast Bacillus
AHR	Aryl Hydrocarbon Receptor
AKT1S1	Proline-rich AKT1 substrate 1
AMBRA1	Autophagy and Beclin 1 Regulator 1
AMK	Amikacin
AMPK	AMP-activated protein kinase
ATGs	Autophagy-related genes
BAD	BCL2 associated agonist of cell death
BAK1	BCL2 antagonist/killer 1
BAL	Bronchoalveolar lavage
BCG	Baccille Calmette-Guérin
BCL2L1	BCL2 like 1
BECN1	Beclin 1
BSL-3	Biosafety level 3
CAP	Capreomycin
CCL4	Chemokine (C-C motif) ligand 4
CDC	Centre for Disease Control and Prevention
CDK4	Cyclin-dependent kinase 4
CDKN1B	Cyclin-dependent kinase inhibitor 1B
CDKN2A	Cyclin-dependent kinase inhibitor 2A
cDNA	Complementary DNA
CFP10	Culture filtrate protein 10
CFU	Colony forming unit
CIP	Ciprofloxacin
Ct	Cycle threshold

CTSB	Cathespin B
CTSD	Cathepsin D
DNA	Deoxyribonucleic acid
DUSP9	Dual specificity phosphatase 9
EIF4G1	Eukaryotic translation initiation factor 4 gamma 1
eis	Enhanced intracellular survival
ELISA	Enzyme-linked immunosorbent assay
EMB	Ethambutol
ER	Endoplasmic Reticulum
ERK	Extracellular signal-regulated kinase
ESAT6	Early-secreted antigenic target 6
ESR1	Estrogen receptor 1
FDA	Food and Drug Administration
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
fig	Figure
GAA	Glucosidase alpha, acid
GABARAPL2	GABA receptor-associated protein ligand 2
GAIP	Gα-interacting protein
gDNA	Genomic DNA
GIPC	GAIP-interacting protein C-terminus
HBC	High burden countries
HDAC1	Histone deacetylase-1
HDAC6	Histone deacetylase-6
HGS	Hepatocyte growth factor-regulated tyrosine kinase substrate
HHC	Household contacts
HIHG	Hussman Institute for Human Genomics
HIV	Human Immunodeficiency Virus
HSP90AA1	Heat shock protein 90 alpha family class A member 1
HTT	Huntingtin
IdeR	Iron-independent regulatory protein

IFNα/β	Interferon α/β
IFN-γ	Interferon-γ
IL-1β	Interleukin 1β
IL-4	Interleukin 4
IL-6	Interleukin 6
INH	Isoniazid
IPA®	Ingenuity pathway analysis
IRGA	Interferon gamma release assays
IRGM	Immunity related GTPase M
ITGA3	Integrin alpha-3
ITGB2	Integrin beta-2
KAN	Kanamycin
LAM	Lipoarabinomannan
LAMP1	Lysosome associated membrane protein 1
LB	Lysogeny broth
LC3	Microtubule-associated protein 1A/1B-light chain 3
LCC	Low copy clade
LIR	LC3-interacting region
LIR	LC3-interacting region
LM	Limannan
LR	Lipid rafts
LRG47	IRGM orthologue
LTBI	Latent TB infections
M. africanum	*Mycobacterium africanum*
M. avium	*Mycobacterium avium*
M. Smegmatis	*Mycobacterium Smegmatis*
M.bovis	*Mycobacterium bovis*
M.canetti	*Mycobacterium canetti*
M.caprae	*Mycobacterium caprae*
M.microti	*Mycobacterium microti*
M.mungi	*Mycobacterium mungi*

M.origys	*Mycobacterium origys*
M.pinnipedi	*Mycobacterium pinnipedi*
M.tb	*Mycobacterium tuberculosis*
mAGP	Mycolic acid-arabinogalactan-peptidoglycan
ManLAM	Mannose-capped LAM
MAPK14	Mitogen-activated protein kinase 14
MBL	Mannose binding lectin
MBT	Mycobactin
MDP	Muramyl dipeptide
MDR	Multidrug resistant
MDR-TB	Multidrug resistant TB
MHC	Major Histocompatibility Complex
min	Minutes
miRNA	MicroRNA
mL	Milliliter
mm	Millimeter
MmpLs	Protein machinery
MOI	Multiplicity of infection
mRNA	Messenger RNA
MTBC	*Mycobacterium tuberculosis* complex
MTOC	Microtubule-organising centre
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
N/A	Not available
ng	Nanogram
NLRs	NOD-like receptors
NOD	Nuclear oligomerisation domain
NRAMP1	Natural resistance-associated macrophage protein 1
NSF	N-ethylmaleimide-sensitive factor
NTF	Noise transfer function
OD	Optical density

OFX	Ofloxacin
P. aeruginosa	*Pseudomonas aeruginosa*
P2RX7	Purinergic receptor P2X 7
p62	Adaptor protein
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PDIM	Phthiocerol dimycocerosate
PE	Phosphaditylethanolamine
PGG	Principle Genetic Group
PGRMC1	Membrane-associated progesterone receptor component 1
PI3K	Phosphoinosisitide 3-kinase
PI3KC3	Phosphatidylinositol 3-kinase catalytic subunit type 3
PIK3CD	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit delta isoform
PIM	Phosphatidyl inositol mannoside
PMA	Phorbol 12-Myristate 13-Acetate
PRRs	Pattern recognition receptor proteins
PtdIns3*P*	Phosphatidylinositol 3-phosphate
PZA	Pyrazinamide
QFT-GIT	QuantiFERON®–TB Gold In-Tube test
RAB24	Member of the RAS oncogene family
RGS19	Regulator of G-protein signaling 19
RIF	Rifampicin
RIN	RNA intergrity number
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
rps30	Ribosomal protein small subunit 30
RPS6KB1	Ribosomal protein S6 kinase beta-1
RT qPCR	Real-time quantitative polymerase chain reaction
S6	Ribosomal protein S6

SA	South Africa
SAC	South African Coloured
sec	Seconds
SFU	Spot-forming units
SL	Sulfolipids
SNPs	Single nucleotide polymorphisms
SQSTM1	Sequestosome 1
STBs	Smooth tuberculous bacilli
TB	Tuberculosis
TBM	TB meningitis
TDM	Trehalose 6,6'-dimycolate
TDM/TMM	Trehalose dimycolate/monomycolate
TDT	Transmission disequilibrium test
TGA3	Transcription factor TGA3
TGFB1	Transforming growth factor beta 1
TH1	Type 1 T helper
THBS1	Thrombospondin-1
TLRs	Toll-like receptors
TMEM74	Transmembrane protein 74
TNF-α	Tumor necrosis factor alpha
T-Spot	T-SPOT®.TB test
TST	Tuberculin skin test
UBA	Ubiquitin-associated
ULK	UNC51-like kinase
USA	United States of America
UVRAG	UV radiation resistance associated
v	Version
V	Volts
VDR	Vitamin D (1,25- dihydroxyvitamin D3) receptor
WHO	World Health Organisation
WIPI1	WD repeat domain, phosphoinositide interacting 1

WNT5A	Wnt Family Member 5A
www	World Wide Web
XDR	Extreme drug resistant
ZN	Ziehl Neelsen

Chapter One

Introduction

CHAPTER ONE

INTRODUCTION

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INTRODUCTION

1.1 TUBERCULOSIS – A GLOBAL EMERGENCY

1.1.1 Tuberculosis epidemiology

Tuberculosis (TB) remains an important global health concern with approximately one third of the world's population infected with the infectious agent, *Mycobacterium tuberculosis* (*M.tb*). Together with HIV, tuberculosis, is the leading cause of death globally due to an infectious agent (World Health Organisation, 2015; Riley, 2015). In 2013, nine million new cases of TB and 1.5 million TB deaths were reported (World Health Organisation, 2014), where 1.1 million of the TB deaths were HIV-negative individuals and 0.4 million HIV-positive individuals (World Health Organisation, 2014). In figure 1.1 below, the estimated global prevalence of TB is shown for 2014.

South Africa is one of 22 TB high burden countries (HBC) that are collectively responsible for approximately 82% of all estimated incident cases worldwide (World Health Organisation, 2014). Worse still, South Africa is one of six countries that had the largest number of incident cases in 2013. According to the World Health Organisation (WHO) Global TB Report 2015, South Africa had 295 477 new TB cases in 2014 with an incidence rate of 834 cases per 100 000 population, and a TB prevalence rate of 696 per 100 000 population (World Health Organisation, 2015). More alarmingly, in 2006, the WHO identified South Africa as having the second highest number of multidrug resistant TB (MDR-TB) cases worldwide (World Health Organisation, 2013). Possible reasons for the high incidence rates in South Africa are due to it being a developing country (Odone et al., 2011) with unfavourable socio economic factors (Hermans et al., 2015) such as low education, low income, a lack of social support, financial problems and not being able to afford services (Muture et al., 2011). Over the last century, there has been a decline in incidence of TB in industrialised countries, however, the increase in immigration of individuals from high burden countries has recently contributed to a reversal of this downward trend (Odone et al., 2011).

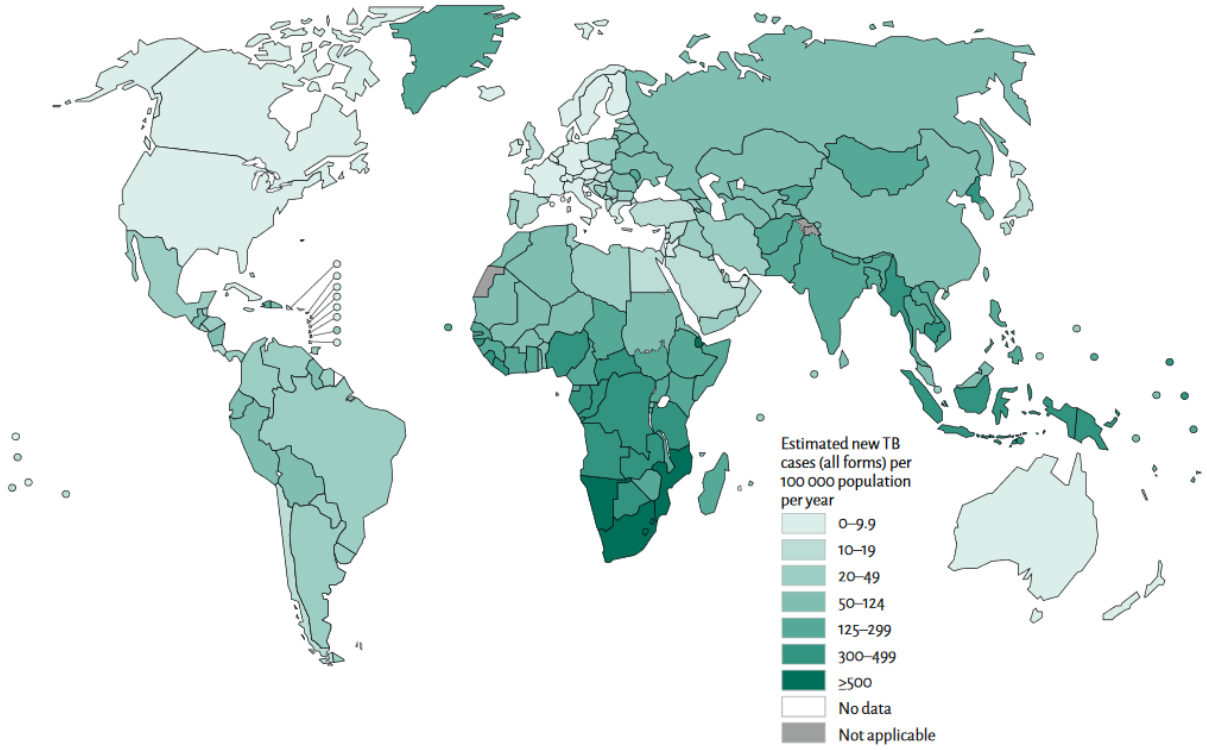

Figure 1.1. The estimated global prevalence of tuberculosis in 2014 (World Health Organisation, 2015).

1.1.2 *MYCOBACTERIUM TUBERCULOSIS* (*M.tb*), CAUSATIVE AGENT

1.1.2.1 The bacteria

In humans, tuberculosis is mostly caused by members of the *M.tb* complex (MTBC), most notably, *M.tb*. *Mycobacteria tuberculosis* is a gram-positive, acid-fast, rod-shaped bacterium (fig. 1.2) belonging to the family *Mycobacteriaceae* (Brighenti and Lerm, 2012). This aerobic family comprises of one genus *Mycobacterium* which has over 150 species and are found in a range of natural environments (Lory, 2014). It is a slow growing mycobacterium with a 12- to 24-hour division rate, that exclusively infects humans (Sakamoto, 2012a; Kaufmann, 2006). A possible reason this family of mycobacteria have managed to survive and flourish is because of their specialised cell wall (Sakamoto, 2012a).

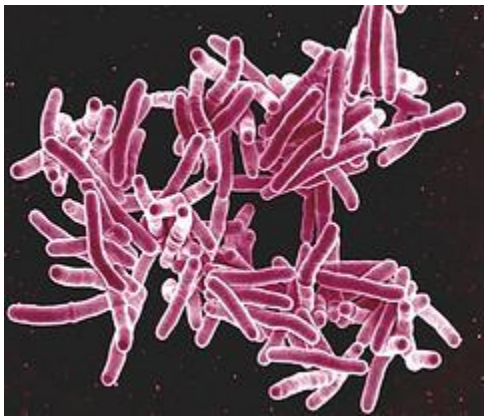

Figure 1.2. The rod-shaped bacteria which is the cause of tuberculosis (National Institute of Allergy and Infectious Disease, 2012).

1.1.2.2 Cell wall

Mycobacterium tuberculosis contains a thick, waxy cell wall which contributes to its success and prevents dehydration, provides protection against various levels of acidity and the damaging effects of free radicals (Wolfe et al., 2010). Furthermore the cell wall contains many proteins (Wolfe et al., 2010) [which includes big amounts of lipoproteins, like beta-hexosaminidase A, preserved membrane proteins, and antigenic proteins like antigen 84/wag31 (Mawuenyega et al., 2005)] and nonproteinaceous antigens (Wolfe et al., 2010) (where mycolic acid, a cell wall component of *M.tb*, presented by CD1b was the first described nonprotein antigen (Flynn and Chan, 2003)), which functions to either stimulate and/or suppress the immune response of the host by being secreted into the extracellular environment. These secreted antigens contributes to the difficulties in treating TB with antibiotics (Wolfe et al., 2010).

The mycobacterial cell wall and its macromolecular features, together with the arabinogalactan core and mycolic acid, have been studied for decades (Wolfe et al., 2010). In figure 1.3 the structure of the cell wall is schematically represented with *M.tb* having a unique inner core made up of mycolic acid-arabinogalactan-peptidoglycan (mAGP) (Wolfe et al., 2010). Furthermore, the cell wall consists of covalently attached carbohydrates and lipids, as well as lipoglycans, free lipids and phophotidyl inositols that are located in the outer core and play critical roles in the modulation of the host immune

(Wolfe et al., 2010). The cell wall specific molecules known to assist in avoiding the host immune response are lipmannan (LM), lipoarabinomannan (LAM), and phosphatidyl inositol mannoside (PIM). Furthermore, the virulence lipids which include, sulfolipids (SL), phthiocerol dimycocerosate (PDIM), and trehalose dimycolate/monomycolate (TDM/TMM) and the protein machinery (MmpLs) are also embedded in the cell wall and are necessary for the export of virulence factors out of the cell (Wolfe et al., 2010).

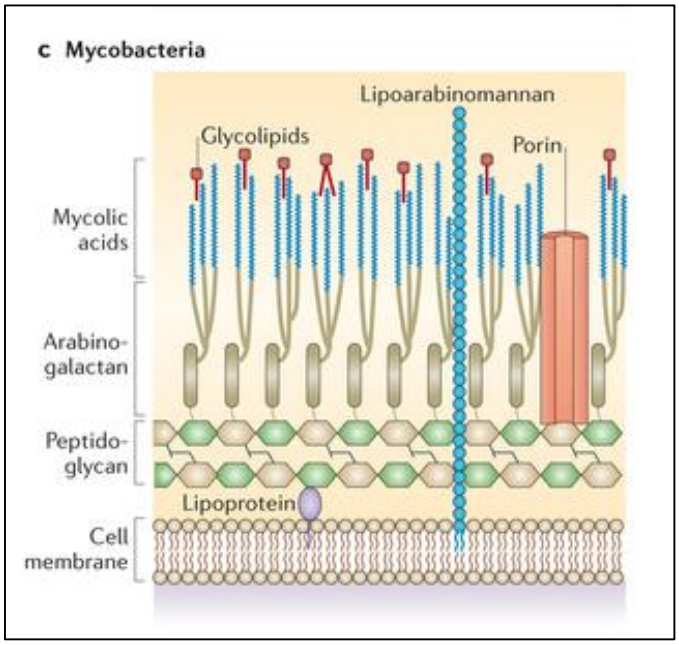

Figure 1.3. The cell walls of mycobacterium contain thin layers of peptidoglycan and arabinogalactan, and a dense layer of mycolic acids. Porins, glycolipids and lipoarabinomannan are also found in the cell walls. The lipoarabinomannan is anchored to the cell membrane by diacylglycerol. The cell wall further surrounds a single lipid membrane (Brown et al., 2015).

1.1.2.3 *Mycobacterium tuberculosis* strains

As mentioned in section 1.1.2.1, *M.tb* consists of a complex (MTBC) which contains a diversity of species that can not only infect humans but animals as well. These members are further divided into different strains, which occurs across the globe in varying prevalence.

The MTBC comprises a large group of closely related bacterial species and subspecies that includes the human adapted *M.tb* and *M. africanum,* and several animal adapted strains such as *M.bovis* (vaccine strain), *M.microti, M.pinnipedi, M.caprae, M.mungi, M.origys,* the dassie bacillus and the chimpanzee bacillus (Smith et al., 2006). Additionally, the complex also comprises more distantly related members that include *M.canetti* and the smooth tubercle bacilli (STBs) (Smith et al., 2006).

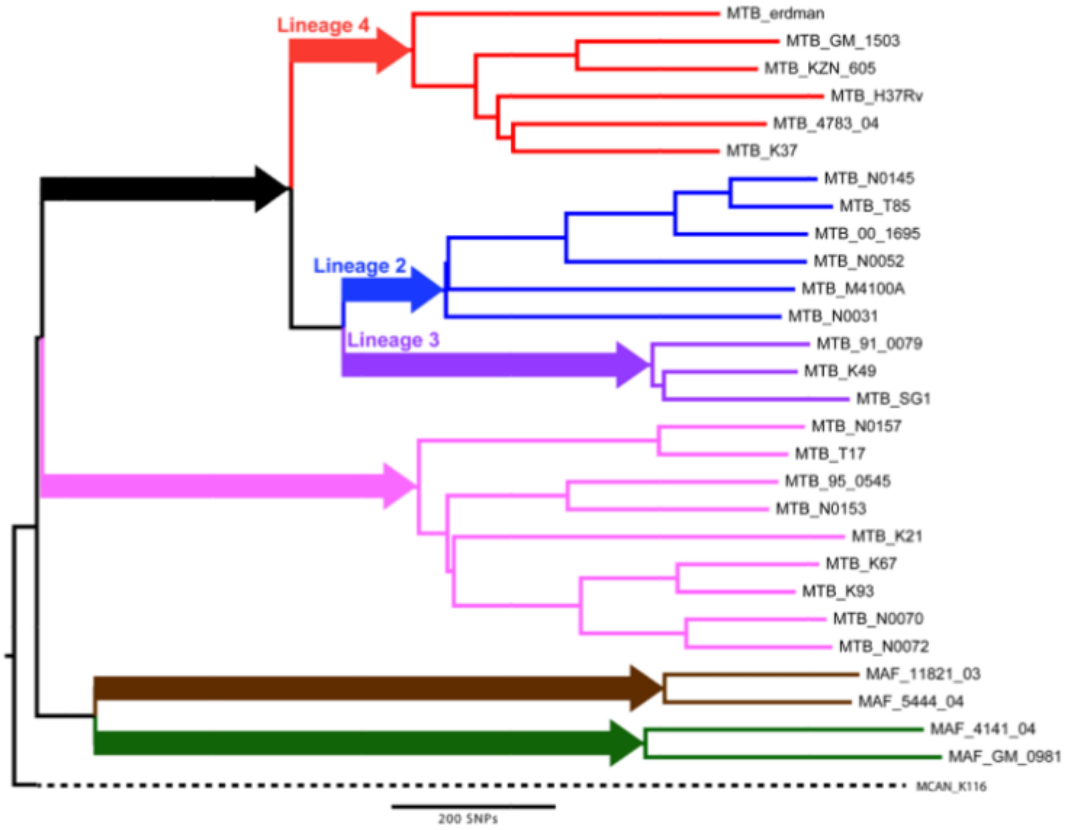

Figure 1.4. A phylogenic tree of *M.tb* which indicates the evolution of the bacterium. The seven main lineages are indicated with bold coloured arrows and a dotted line (Coscolla and Gagneux, 2014). The phylogenic tree is split up into two, namely evolutionary "ancient" - and "modern" lineages. The three modern lineages diverged separately and at a later time point than the ancient lineages which branched off from a common ancestor at an earlier stage of evolution (Portevin et al., 2011). It is believed that the diversions are caused by human migration out of Africa, with the expansion and migration of the different lineages being determined by their host populations (Portevin et al., 2011).

One of the very first attempts to reconstruct the genetic evolution of the MTBC identified a group of strains that contained a deletion in a genome region designated TbD1. These TbD1-deletion strains are referred to the evolutionary “modern strains”, while those without the TbD1 deletion are referred to as the “ancestral” or “ancient” strains (Brosch et al., 2002b).

With the development of next generation sequencing technologies, the ability to track the evolutionary history of MTBC strains has improved considerably. Based on comparative analyses of the genomes of members of the MTBC, we are now able to subdivide the MTBC into seven human adapted lineages, namely lineage 1 through 7 (fig 1.4). Based on comparative genomics, the “modern” clade form a monophyletic group comprising lineages 2, 3 and 4, while the “ancient” strains, by contrast are paraphyletic which means they encompass more than one phylogenetic group (Coscolla and Gagneux, 2014).

The human-adapted MTBC lineages exhibit a strong phylo-geographical population structure, with some lineages found to be associated with distinct geographical regions (Baker et al., 2004; Brosch et al., 2002b; Filliol et al., 2006; Gagneux et al., 2006; Hirsh et al., 2004; Hershberg et al., 2008), while others are more geographically wide-spread (Coscolla and Gagneux, 2014). Lineages 1 and 3 are quite restricted geographically as they are mostly limited to East Africa, Central Asia, South Asia and Southeast Asia. Lineages 5 to 7 are the most geographically restricted lineages and are mainly confined to specific regions in Africa. Lineages 5 and 6 are exclusively found in West Africa, while lineage 7 is confined to Ethiopia (Coscolla and Gagneux, 2014).

Lineage 2 and 4 are the most geographically widespread of all the lineages. Lineage 2 is the dominant lineage in East Asia, but is also highly prevalent in Central Asia, South Africa, Vietnam and Russia. This lineage is one of the most successful MTBC variants; more than 25% of the global TB epidemic is caused by this lineage. Furthermore, lineage 2 contains strains that belong to the Beijing strain family (Hershberg et al., 2008; Comas et al., 2009), a family that has been the focus of much research because of its tendency to cause disease outbreaks and its association with resistance to anti-mycobacterial

drugs (Parwati et al., 2010). It is thought that the Beijing strain family evolved mechanisms that allow for the evasion of the protective immune response induced by the BCG vaccine, enable efficient transmission and development of anti-mycobacterial drug resistance (Aguilar L et al., 2010).

In Vietnam, Russia and South Africa the Beijing strain family is dominant among the local circulating strains (Lasunskaia et al., 2010). In Russia alone the Beijing strain comprises half of the individuals infected with *M.tb*, and at least 80% of these strains have been found to be resistant to at least one of the anti-TB drugs, while 65% were MDR (Lasunskaia et al., 2010).

The Beijing strain can be sub classified as 'typical' or 'atypical' depending on the presence or absence of an *IS6110* insertion in the noise transfer function (NTF) region (Klopper et al., 2013). The ability of the Beijing strain to gain dominancy through replacing resident strains has been shown in a community-based study in Cape Town, South Africa where the Beijing strain were tracked over a period of 12 years (Marais et al., 2013). Multiple factors can be attributed to the success of the Beijing strain, including resistance to *M.bovis* BCG-induced immunity, possible hypervirulence, and the reduced fitness costs associated with drug resistant acquisition (Marais et al., 2013).

It has been proposed that different *M.tb* lineages have adapted to certain human populations. However, comorbid infections such as HIV could disrupt this geographic sympatric human host-*M.tb* relationship (Middelkoop et al., 2015). It was reported that the Beijing strain has emerged and diversified from East Asia, where it is believed to have originated from (Hanekom et al., 2007), and this phenomenon could well be due to the occurrence of other diseases (Middelkoop et al., 2015).

It was only after 1965 that the Beijing strain emerged in Cape Town. Before then there was no evidence of this strain which now accounts for approximately 20% of all TB cases (Fallow et al., 2010). This led to the assumption that the Beijing strain possess unique attributes that gives it an increased ability to not only cause disease but to transmit to

other regions and cultures (Fallow et al., 2010). A study by Wang et., al. (2011) stated that 19% of all TB patients in the Cape Town region are simultaneously infected with a Beijing and a non-Beijing strain, and that this number increased to 23% in retreatment cases. Another study reported that the Beijing strain was dominant in children from Cape Town with confirmed TB culture, whereas the dominant strain among adults in the same region was the LAM strain and this could be due to an important "age-shift" as seen in Vietnam as well (Marais et al., 2006). The success of the resident strains could be attributed to the virulence of the strain types.

1.1.3 Virulence of *Mycobacterium tuberculosis*

There is long-standing evidence showing that some *M.tb* strains are more virulent than others (Nicol and Wilkinson, 2008). This phenomenon was first described over 50 years ago when Mitchison and colleagues (1960) showed that *M.tb* strains from southern India were less virulent in guinea pigs than strains from the United Kingdom. More recently, studies have demonstrated a large degree of variation in virulence of *M.tb* strains following infection of mice and rabbits (Dunn and North, 1995; Miyazaki et al., 1999; Lopez et al., 2003; Dormans et al., 2004; Aguilar et al., 2010).

There is also mounting evidence which suggests that even within strain families there are varying degrees of virulence amongst members. For instance, comparative survival studies in murine models have shown that *M.tb* strains of the Beijing genotype had higher levels of virulence compared to non-Beijing genotypes when using time to death and organ bacterial load as proxies for the level of virulence (Manca et al., 2001; Lopez et al, 2003; Roberts et al., 2007). Interestingly, in a follow-up study by Dormans and colleagues (2004), they investigated three Beijing genotype strains and found that two of these strains were highly virulent, while the other strain was less virulent, suggesting that not all Beijing genotype strains were hyper-virulent. This was further highlighted with the study of Aguilar and co-workers (2010) that evaluated the levels of virulence amongst members of the seven phylogenetically determined (Hanekom et al., 2007) sublineages of the *M.tb* Beijing strain. In their investigation, these researchers infected BALB/c mice with Beijing strain representatives of the different lineages and of different epidemiological

characteristics (transmitted or not transmitted) and used survival times, lung pathology, bacterial load and immunology kinetics to define virulence. They showed that mice infected with highly transmitted Beijing genotypes only survived for five weeks post-infection, suggesting hyper-virulence, while more than 80% of mice infected with non-transmitted strains survived for four months post-infection, suggesting a low virulence. From this data, it is clear that even in strains, different members of strain sublineages show different levels of virulence (Aguilar L et al., 2010).

The significance of these findings in the context of human *M.tb* is still relatively uncertain, but several lines of evidence have emerged which suggests that different *M.tb* strains elicit different immune responses. In a study of 26 global clinical isolates, representing six *M.tb* lineages, considerable heterogeneity in the inflammatory response induced by these strains was observed. Strains from the "modern" lineages (Euro-American, Beijing, and India/East Africa) induced less of an inflammatory response compared to those from the "ancient" lineages (Indo-Oceanic and West Africa) (Portevin et al., 2001). It is hypothesized that this decreased inflammatory response may confer a selective advantage to the "modern" strains as it results in impaired bacterial control by the host leading to more rapid progression to disease and enhanced transmission. Furthermore, upon comparing 187 *M.tb* strains isolated from cerebrospinal fluid of adults with TB meningitis to 237 strains isolated from sputum of adult patients suffering from pulmonary TB, strains from the Euro-American lineage were observed to be less likely to cause TB meningitis than strains from the Indo-Oceanic or Beijing lineages (Caws et al., 2008). In order to make sense of their observations, researchers characterised representatives from each of the lineages in macrophages, dendritic cells and in mice and found that the Beijing strains disseminated more rapidly in the blood compared to the Euro-American strains (Krishnan et al., 2011). Moreover, Beijing and Indo-Oceanic strains induced significantly more tumor necrosis factor alpha (*TNF-α*) and interleukin 1β (*IL-1β*) compared to Euro-American strains (Krishnan et al., 2011). Results from these studies provide persuasive evidence for strain specific differences in virulence and induction of host immune responses. However, the mechanisms by which these varying responses are induced remains largely unclear.

1.2 TUBERCULOSIS (TB) PATHOGENESIS

Infections typically begin by inhalation of aerosol droplets containing approximately 1 - 200 *M.tb* bacilli from an individual with “open” pulmonary disease (Kaufmann, 2002; Sakamoto, 2012b). The spread of the aerosol droplets are aided by coughing, talking or sneezing and these particles can linger in the air for long periods of time (Curry, 2007). The lung has a dual purpose of serving as the site of entry and the primary site of infection (Kaufmann, 2002).

Once the pathogen is recognised by alveolar macrophages, the innate immune response is activated (Kaufmann, 2002; Magee et al., 2014) and the bacilli are rapidly phagocytosed (Sakamoto, 2012b), which is critical for host defense against *M.tb* (Magee et al., 2014). This recognition occurs through the interaction of pathogen-associated molecular patterns (PAMPs) with cell surface pattern recognition receptor proteins (PRRs), which includes Toll-like receptors (TLRs) (Magee et al., 2014). After the mycobacterial-induced TLR signaling is activated it triggers a range of intracellular pathways that leads to the production of endogenous TH1 (type 1 T helper) inflammatory chemo and -cytokines (Magee et al., 2014) such as Chemokine (C-C Motif), Ligand 4 (CCL4), *IL-1β*, interleukin 6 (*IL-6*) and *TNFα*, that recruits cytotoxic T cells to the site of infection. Additionally, these chemo- and cytokines drive the recruitment of leukocytes, monocytes and neutrophils to the site of infection leading to the phagocytosis of bacilli as well as the formation of an early granuloma (Sakamoto, 2012b).

Furthermore, *M.tb* is also phagocytosed by dendritic cells that subsequently migrate to regional lymph nodes to present the mycobacterial antigens to lymphocytes (Sakamoto, 2012b). Ultimately a granuloma will develop that consists of infected macrophages and monocytes, which are surrounded by epitheloid macrophages, foam cells, and occasionally multinucleated giant cells (Langerhans cells), peripheral recruited lymphocytes and a fibrous capsule (figure 1.5) (Sakamoto, 2012b). These granulomas, together with antigen-specific T cells are key host defense mechanisms against TB infection (Kaufmann, 2002). The granulomas, which contain the mycobacteria, focus the

immune response to a certain area and decrease the spread of the invading mycobacteria (Guirado and Schlesinger, 2013). It is therefore vital that the granulomas remain intact; impaired formation has been linked to increased disease severity (Kaufmann, 2002). If granulomas are functioning optimally, the bacillus can be contained for long periods of time and not cause active disease.

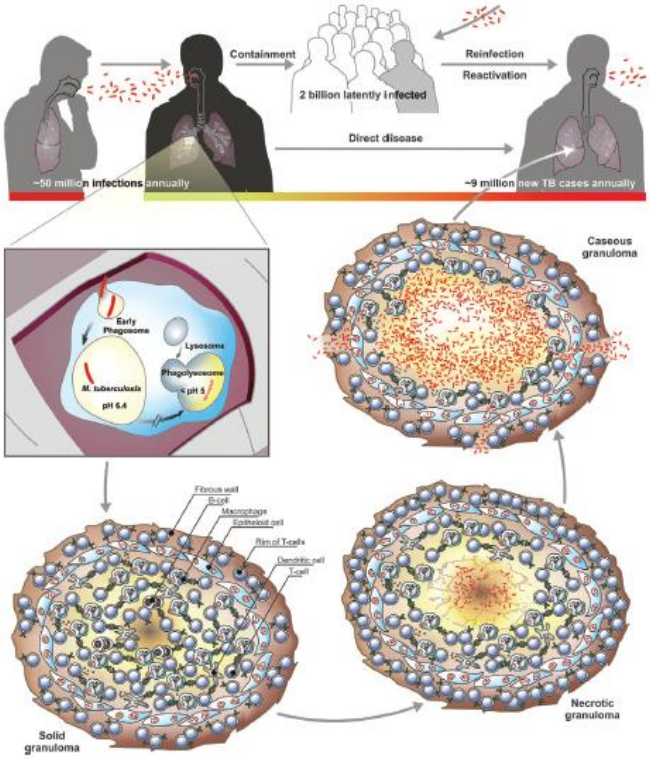

Figure 1.5. The pathogens' route of infection - from transmission between individuals and granuloma formation to infecting other individuals (Gengenbacher and Kaufmann, 2012).

1.2.1 Latent TB infection

It has been estimated by WHO that only 5-10% of TB infected individuals will develop active TB disease (Dittrich et al., 2015), while the rest remain latently infected. Individuals with latent TB infections (LTBI) can remain healthy for long periods of time, showing no signs or symptoms of active TB disease. These individuals however, will have an increased risk of developing active TB disease in their lifetime (Hur et al., 2013). Individuals who are exposed or infected with *M.tb* have memory T cells which will respond promptly when exposed to mycobacterial antigens once more (Carpenter et al., 2016).

The tuberculin skin test (TST) or interferon gamma release assays (IGRA) are used to diagnose LTBI, and research is leaning towards treating LTBI and preventing it from turning into active disease (Diel et al., 2012).

1.2.2 Diagnosis and Treatment

1.2.2.1 Diagnosis

There are several diagnostic tests available for TB. These include Quantiferon (Ferrara et al., 2006), Ziehl Neelsen (ZN) stains (Cain et al., 2010), GeneXpert (Boehme et al., 2011), chest radiographs (figure 1.6) and bacterial culture which still remains the gold standard (Cain et al., 2010; Pasco, 2012). The different tests for diagnosing TB, together with their functions are listed in table 1.1.

Table 1.1. Type of methods and their function to test for patients TB status (Knechel, 2009; Theron et al., 2014).

Type of test	Function of the test	Time required to obtain results
Sputum smear	Detect acid-fast bacilli	<24 hours
Sputum culture	Identify *M.tb*	•3-6 weeks with solid media •4-14days with high-pressure liquid chromatography
Polymerase chain reaction	Identify *M.tb*	Hours
Tuberculin skin test	Detect exposure to mycobacteria	48-72 hours
Quantiferon -TB test	Measure immune reactivity *M.tb*	12-24 hours
Chest radiography	Visualise lobar infiltrates with cavitation	Minutes

Type of test	Function of the test	Time required to obtain results
Xpert MTB/RIF	Detects TB and RIF resistance	Less than 2 hours

The primary TB diagnostic method in low and middle income countries has been sputum smear microscopy (Desikan, 2013), mainly because it is rapid, simple, inexpensive and highly specific in areas where the TB prevalence is high. However, this method does have its limitations which include lowered sensitivity when the bacterial load is 10,000 organisms/mL sputum sample or less and decreased effectiveness in paediatric patients, patients with extra-pulmonary TB and patients who are co-infected with HIV (Desikan, 2013; Theron et al., 2014).

The tubercle bacilli are identified in sputum smears through ZN staining (Chandra et al., 2014). Although acid-fast microscopy is quick and easy, it is not a definite confirmation of the TB disease. Therefore, a culture is done to confirm the results. If the culture is positive for *M.tb* it is a definite indication of TB disease, thus it should be done regardless of the results obtained from the AFB (Acid-Fast Bacillus) smear (Pasco, 2012).

For more than a century, the TST was the only available test to detect LTBI (Ramos et al., 2012). One major limitation of this method, however, is a decreased sensitivity in patients who are HIV-infected compared to the general population, especially in those whose CD4 cell count is low. Testing of TB with the TST entails the individuals being injected with 0.1mL tuberculin. Tuberculin is sterile protein extracts of tubercle bacillus cultures. The skin induration is measured at 48-72 hours after the injection and the individual is considered positive if the size of the induration is 5mm or more (Ramos et al., 2012).

With the recent development of *in vitro* blood tests, such as Interferon gamma release assays (IGRAs) which evaluate cell-mediated immune responses against *M.tb,* the diagnosis of LTBI has become easier. Interferon release assays measure the release of

interferon-γ (*IFN-γ*) by T-cells following stimulation with *M.tb*-specific antigens such as the early-secreted antigenic target 6 (*ESAT6*) and culture filtrate protein 10 (*CFP10*). These antigens are significantly more specific to *M.tb* than the antigens present in the purified protein derivative used in the TST since they are not shared with any of the Bacilli Calmette-Guérin (BCG) vaccine strains (Ramos et al., 2012).

The Quantiferon ®–TB Gold In-Tube test (QFT-GIT) and the T-SPOT®.TB test (T-Spot) are two IGRAs approved by the USA Food and Drug Administration (FDA) and are preferred for individuals who have received the BCG vaccine or those who are unable to return for a second appointment to evaluate TST response. The QFT-GIT test measures the level of soluble IFN-γ in whole blood from patients through an enzyme-linked immunosorbent assay (ELISA), while the T-Spot assay assesses spot-forming units (SFU) which is representative of the number of IFN-γ–producing cells (Ramos et al., 2012). It should be noted that a positive result from a TST or IRGA result only confirms the presence of *M.tb*; it is unable to determine whether the individual has LTBI or if it has progressed to active TB disease (Centers for Disease Control and Prevention, 2014).

The Xpert MTB/RIF is an automated nucleic-acid amplification test able to detect *M.tb* complex DNA within two hours. Additionally, this test is able to determine whether the infecting strain is resistant to rifampicin, one of the first-line drugs for TB treatment (see section 1.4.2), and can detect resistance within 2 hours (Theron et al., 2014). The accuracy of this method is well confirmed; one Xpert MTB/RIF assay detects on average 88% of confirmed pulmonary TB culture cases, correctly determines about 98% of individuals without TB, and detects 67% of cases which were missed by smear microscopy. Furthermore, this assay can identify 94% of rifampicin-resistant TB cases and accurately identify 98% of rifampicin-susceptible cases (Theron et al., 2014). Once TB is confirmed (either active or latent) using one of the methods listed in table 1, an optimal treatment plan is structured for the patient.

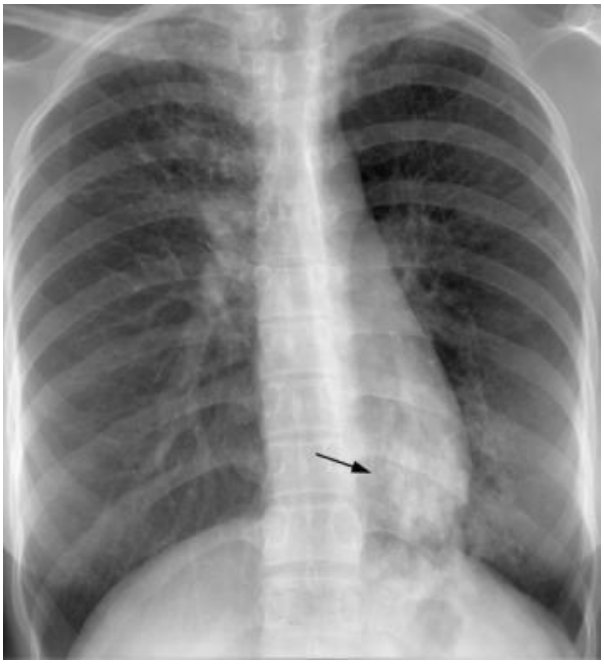

Figure 1.6. A chest x-ray of a young male patient who presented symptoms typical of tuberculosis. In the lower left lobe a focal opacity, indicated with an arrow, is seen which is indicative in cases of primary tuberculosis in adults (Catanzano, 2016).

1.2.2.2 Treatment

The standard regimen for new TB patients is divided into phases (WHO | Guidelines for treatment of tuberculosis). During phase one, patients receive daily doses of the first line anti-tuberculosis drugs isoniazid (INH), rifampin (RIF), pyrazinamide (PZA) and ethambutol (EMB) (Campbell et al., 2011). This phase is the intensive phase and lasts for two months. For smear-negative pulmonary TB patients, patients with TB meningitis or extra-pulmonary TB patients who are HIV negative, EMB is replaced by streptomycin during the intensive phase. Pyrazinamide is responsible for disrupting the plasma membrane and the energy metabolism, Ethambutol and Isoniazid both act on inhibiting cell wall synthesis, and Rifampicin is responsible for inhibiting RNA synthesis (Bordons, 2013). The second phase of treatment is the continuation phase which lasts for four months. During this phase, patients receive daily or thrice-weekly doses of INH and RIF (WHO | Guidelines for treatment of tuberculosis).

The emergence of drug resistant *M.tb* strains has led to the introduction of several second line anti-TB drugs which are more expensive and can lead to more unfavourable side effects. These second line drugs, which include amikacin (AMK), capreomycin (CAP), kanamycin (KAN), ciprofloxacin (CIP), and ofloxacin (OFX), are used when drug susceptibility testing of infecting strains show resistance to any of the first line drugs. Unfortunately, multi- and-extensively-resistant (MDR and XDR) strains have also recently emerged. The Centre for Disease Control and Prevention (CDC) and WHO in 2006 defined MDR-TB as a form of TB that is resistant to treatment with at least two of the front-line anti-TB drugs, while XDR-TB has been defined as resistant to RIF and INH in addition to any fluoroquinolone (CIP and OFX) and at least one of the second-line injectable drugs (AMK, CAP and KAN) (Campbell et al., 2011).

Latent TB infection is an important reservoir for new TB disease and for the spread of *M.tb* within communities. Given that one third of the world's population is latently infected with *M.tb*, effective treatment of LTBI is essential for eradicating TB globally (Salgame et al., 2015). Currently, the standard regimen for the treatment of latent *M.tb* infection is daily INH monotherapy for six to nine months. Adherence to and completion of a six month and 12 month regimen of INH monotherapy has been shown to reduce the risk to LTBI progression to active TB disease by 69% and 93% respectively (Bull World Health Organ, 1982). It should be noted that these statistics are from a study conducted in 1982, before the era of widespread HIV infection. Additionally, owing partly to long duration rates of the regimen, the completion rates of 30% to 64% limits the effectiveness of INH monotherapy for LTBI treatment (American Thoracic Society, 1999; LoBue & Moser, 2003; Horsburgh et al., 2010; Horsburgh & Rubin, 2011). Another regimen consisting of pyrazinamide and rifampicin taken for 2 months has been shown to be as effective as INH monotherapy for LTBI treatment, however, due to its increased rates of severe chemical-induced liver damage, it is not recommended (Sterling et al., 2011).

1.2.3 Evasion of the host immune response by *M.tb*

As stated in section 1.1.2, following inhalation, alveolar macrophages engulf the *M.tb* bacillus using phagocytic receptors, such as the complement and mannose receptors (Behar et al., 2011). Virulent *M.tb* is able to survive within the host by preventing phagosomal compartment acidification and impairing phagolysosomal fusion (Kaufmann, 2002; Behar et al., 2011). *Mycobacterium tuberculosis* can thus adapt to its intracellular environment (Behar et al., 2011) by changing the behavior of macrophages and their surrounding tissue (Podinovskaia et al., 2013). It is interesting to note that both innate and adaptive immunity is able to modulate the course of *M.tb* infection, however, this microbe has developed cunning mechanisms to evade host immune responses. In the section that follows, the mechanisms by which *M.tb* evades the host immune response will be discussed. It should however be noted that since the study presented in this thesis focuses on the role of autophagy in TB susceptibility that the evasion of the host innate and adaptive immunity will be briefly discussed, while a more in depth discussion of autophagy will be presented.

1.2.3.1 Evasion of the innate immune system

The innate immune system, particularly the cellular arm, is reliant on a variety of pattern recognition receptors comprising members of the TLRs, nuclear oligomerisation domain (NOD) and NOD-like receptors (NLRs), complement receptor, C-type lectin receptor and mannose receptor families (Coll and O'Neill, 2010). Each of these receptor families play crucial roles in recognition of *M.tb* and in its uptake by phagocytic cells.

Mycobacterium tuberculosis expresses a number of diverse lipoprotein and lipoglycan receptor moieties on the surface of its cell wall that is recognised by TLR2 heterodimeric complexes resulting in TLR2-dependant macrophage activation, cytokine production and granuloma formation. Lipoarabinomannan (LAM) from non-pathogenic mycobacterial cell walls is a highly immunogenic TLR2 agonist, while the mannose-capped LAM (ManLAM) from virulent mycobacteria is not. Binding of LAM to TLR2 results in the initiation of *IFN-γ* receptor signaling in macrophages, the production of L-12p70 by dendritic cells,

phagosomal maturation and apoptosis of *M.tb* infected cells. However, the mannose-capped LAM on the surface of pathogenic mycobacteria confounds the ability of LAM to initiate these aforementioned processes and it is therefore thought that *M.tb* has used this modification to subvert the TLR2-mediated signaling (Briken et al., 2004; Goldberg et al., 2014).

Trehalose 6,6'-dimycolate (TDM) is another mycobacterial cell wall glycoprotein that is recognised by host innate immune receptors, including the macrophage C-type lectin (Mincle) (Morita et al., 2013). Recognition of TDM by Mincle leads to macrophage activation resulting in the secretion of proinflammatory cytokines (Ishikawa et al., 2009). In order to avoid detection by Mincle, pathogenic mycobacteria have evolved a mechanism by which it is able to down-regulate TDM expression. Mycobacterial mycolytransferases catalyze the last step in the biosynthesis of TDM from trehalose 6-monomycolate. However, upon entry into the host, virulent mycobacteria, such as *M.tb* uses host-derived glucose as competitive substrate for the enzymes which leads to the down-regulation of TDM synthesis (Matsunaga and Moody, 2009).

Electron microscopy studies have eloquently shown that virulent mycobacteria are periodically able to escape from phagosomes into the cytosol of infected macrophages (McDonough, Kress, & Bloom, 1993a, 1993b; Houben, Nguyen, & Pieters, 2006; van der Wel et al., 2007) where they encounter a range of cytosolic pattern recognition receptors involved in shaping innate and adaptive immunity (Koizumi et al., 2012). One such receptor is NOD2 which senses muramyl dipeptide (MDP) fragments of bacterial peptidoglycan which subsequently results in an immune response. Virulent *M.tb* expresses an N-glycoyl modified form of MDP (as opposed to the N-acetylated form produced by most other bacterial species) that is capable of triggering the production of interferon α/β (*IFNα/β)* in infected macrophages which antagonises the host-protective *INF-γ* and *IL-1β* pathways (Goldberg et al., 2014). Studies by various research teams conducted using type I IFN receptor-deficient mice have shown that *INFα/β* has little effect on the *M.tb* growth inside the lung and may even promote its growth (Juárez et al., 2012; Pandey et al., 2009; Stanley et al., 2007).

1.2.3.2 Evasion of the adaptive immune system

In addition to subverting the innate immune system, pathogenic mycobacteria have evolved a number of mechanisms that limit or manipulate all known pathways of antigen presentation to T-cells (Russell, 1995; Flannagan et al., 2009). Given that mycobacteria can directly and effectively infect antigen presenting cells, this is a major contributing factor to its ability to evade antigen-specific T-cell responses.

The major cell type infected by *M.tb* are macrophages, a cell type that is able to present antigenic peptides to effector T-cells on both major histocompatibility complex (MHC) class I and class II molecules. While the capacity of macrophages to present antigens to previously primed effector or memory T-cells may be crucial for its anti-mycobacterial functions, it is highly unlikely that they are effective in priming naïve T-cell responses against *M.tb*. This function is by and large carried out by highly specialised subsets of dendritic cells that are able to process and present most types of antigens to naïve CD4+ and CD8+ T-cells (Wolf et al., 2007).

Studies using green fluorescently labelled *M.tb* have shown that in addition to infecting alveolar macrophages, *M.tb* is able to infect lung myeloid dendritic cells which then transports mycobacterial antigens to draining lymph nodes, leading to the initiation of an adaptive immune response (Wolf et al., 2007). However, in chronically infected dendritic cells, MHC class II expression may be down-regulated which limits T-cell priming. These chronically *M.tb*-infected dendritic cells that have not been sufficiently activated can serve a reservoir for *M.tb* and acts as a vehicle for mycobacterial dissemination into other tissues and delay the initiation of the adaptive immune response. Dendritic cells have marked migratory potential and infection of these cells plays an important role in development of extrapulmonary TB (Flynn and Chan, 2003; Humphreys et al., 2006).

Mycobacterium tuberculosis has also evolved mechanisms by which it is able to disrupt the maturation of dendritic cells and in doing so evade the host adaptive immune response. Currently there are two models that have been described. Firstly, in two studies using cultured monocyte-derived macrophages, it was shown that following infection with

M.tb, these cells did not mature normally as indicated by the lack of rapid movement of MHC class II molecules to the cell surface (Henderson et al., 1997; Hanekom et al., 2003). In this model, it is postulated that *M.tb* infection results in the blockage of dendritic cell maturation. Secondly, in a study by Hava and colleagues (2008), it is hypothesised that immune evasion occurs not as a result of maturation blockade, but rather as a result of the stimulation of poorly coordinated dendritic cell maturation that causes antigen presentation to stop before the *M.tb* antigen production is initiated. In this model, it is thought that this poor coordination results in the rapid maturation of infected dendritic cells resulting in the movement of a large majority of MHC class II molecules to the cell surface in parallel with the termination of production of new MHC class II molecules. This model therefore suggests by the time that mycobacterial antigens are available for process and antigen presentation, that the majority of MHC class II molecules have already been sequestered to the cell surface which limits the pool of MHC class II molecules available in endocytic compartments for peptide loading (Hava et al., 2008).

In addition to disrupting antigen presentation on dendritic cells, *M.tb* is also able to inhibit MHC class II antigen presentation in macrophages (Pancholi et al., 1993). Two independent investigations have found that fewer macrophages infected with *M.tb* present antigens on their cell surface compared to uninfected macrophages (Kaye et al., 1986; Mshana et al., 1988). Gercken and colleagues also showed that monocytes co-cultured with *M.tb* exhibited up to 10-fold reduction in their capacity to stimulate T-cells when compared to uninfected monocytes (Gercken et al., 1994). These results were later corroborated and evidence of an inverse correlation between *M.tb* infectious dose and T-cell response was shown (Mazzaccaro et al., 1996; Noss et al., 2000).

1.2.3.3 Evasion of autophagy

Autophagy is a catabolic process that degrades undesirable cytosolic components (Deretic, 2011) and can easily be distinguished from other vesicles on electron micrographs since it possesses a double-unit limiting membrane (Haspel and Choi, 2011). Different forms of autophagy exists which includes xenophagy (section 1.2.3.3.2),

chaperone-mediated autophagy (which will not be looked at in this study), macroautophagy (commonly referred to as autophagy) and mitophagy.

The targeted cytoplasmic components are isolated from the rest of the cell within a double-membraned vesicle known as an autophagosome (figure 1.7). Once an autophagosome has engulfed the cargo and matured, the phagosome will co-localize with the autophagy effector microtubule-associated protein 1A/1B-light chain 3 (LC3) (Shin et al., 2010a), and will then fuse its external membrane with late endosomes and lysosomes to form an autophagolysosome (Haspel and Choi, 2011). LC3 is a soluble protein distributed throughout the mammalian tissue and cultured cells (Tanida et al., 2008). While the autophagosome engulfed cytosolic proteins and organelles, the cytosolic form, LC-I, is conjugated to phosphatidylethanolamine, which covalently modifies the protein by lipidation and removal of a short amino acid to form, LC-II, which is recruited to the membranes of autophagosomal membranes (Tanida et al., 2008). LC-II is converted to initiate the formation and elongation of the autophagosome. The autophagolysosomes degrade by lysosomal hydrolases (Tanida et al., 2008) and recycle its cargo, whilst progressively losing their unique membrane structure (Haspel and Choi, 2011). While the cargo is being degraded, LC3-II is also degraded in the autophagolysosomal lumen, thus this autophagosomal marker (LC3-II) reflects starvation-induced autophagic activity (Tanida et al., 2008). The targeted cytoplasmic components are degraded through exposure to a reduced pH, proteases and anti-microbial peptides. This process not only targets proteins, but also lipids, carbohydrates, and entire organelles (for example peroxisomes and mitochondria). The products of digestion are subsequently recycled back to the cytoplasm through lysosome permeases and used in numerous biosynthetic pathways (Haspel and Choi, 2011).

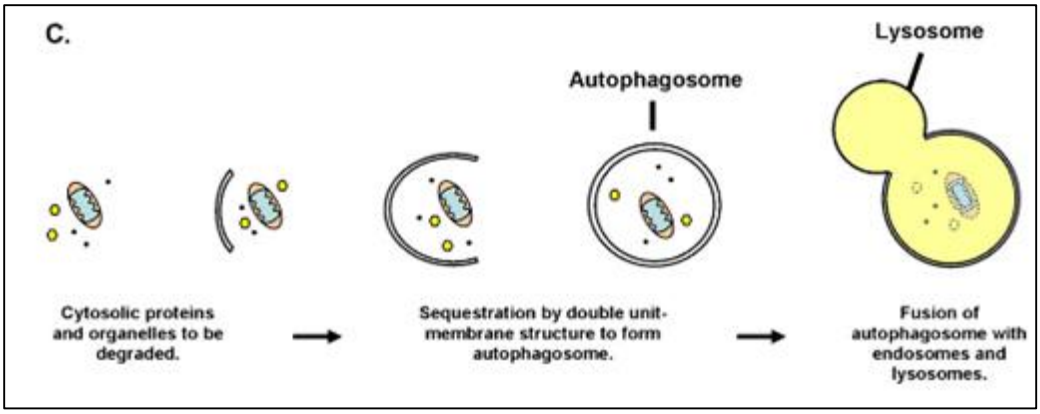

Figure 1.7. Schematic illustration of macroautophagy (Haspel and Choi, 2011). During this process, targeted cytoplasmic components such as proteins and organelles are isolated from the rest of the cell within a double-membraned vesicle known as an autophagosome. Once an autophagosome has matured, it fuses its external membrane with late endosomes and lysosomes to degrade and recycle its cargo, whilst progressively losing their unique membrane structure.

In addition, autophagy-related genes (ATGs) encodes for the majority of proteins in different pathways that help facilitate the autophagy pathway. ATGs are not only responsible for the formation of autophagosomes, but the genes form complexes in order to regulate various steps of the autophagy process, namely initiation, nucleation and elongation. The ATG proteins can be divided into four groups based on their molecular machinery used in the formation of an autophagosome. The first group is the UNC51-like kinase (*ULK*) complex, which consists of the UNC51-like Ser/Thr kinases, *ULK1* and *ULK2, ATG13, FAK* family kinase-interacting protein of 200 kDa (*FIP200*) and *ATG101*. The second group consists of phosphatidylinositol 3-kinase class III (*PI3K*) *Vps34* and *BECN1*, which marks the site of autophagosome generation through increased phosphatidylinositol 3-phosphate concentration. Thirdly, the group *ATG9* and *VMP1* are transmembrane proteins necessary for recruiting membrane for the autophagosome formation; and the fourth group is the two ubiquitin conjugation systems, *ATG12*-like and *MAP1LC3* (Castrejón-Jiménez et al., 2015; Deretic et al., 2015). The above mentioned ATG proteins, together with supplementary ATG factors, lead to the development of an autophagosome from various membrane sources such as endoplasmic reticulum (ER)

and the endosomal system. The autophagosome lengthens, captures cytoplasmic targets reserved for autophagic degradation, and lastly delivers them to lysosome (Deretic et al., 2015).

1.2.3.3.1 The role of autophagy in nutrient starvation conditions

Autophagy also plays a role in nutrient starvation conditions where cellular components obtained from the recycled cargo are used for energy sources. The mammalian target of rapamycin (mTOR) serves as a major autophagy regulator due to the connection between autophagy and metabolism. The mTOR pathway will be activated when nutrients are in abundance, which will lead to the phosphorylation of p70-S6 kinase. Consequently this will lead to the phosphorylation of ribosomal protein S6 (S6) which is one of the numerous mTOR targets that can lead to cell growth and differentiation through up-regulation of protein translation. On the other hand, protein translation can significantly be reduced in conditions of nutrient starvation, due to a reduction in phosphorylation of p70-S6 kinase and S6. Anabolic processes can be shut down if there is a lack of mTOR signaling and therefore facilitates the formation of *LC3B-II*-positive autophagosomes. These autophagosomes will degrade the contents by engulfing cytosolic material and fusing with lysosomes. In summary, mTOR-dependent autophagy has a housekeeping function, which supports cell viability during times of nutrient deficiency (Zullo and Lee, 2012a).

It is also well accepted that the mTOR pathway is an effective way by which intracellular microorganisms can be detected, contained and destroyed. Through experimental assays Zullo and Lee (Zullo and Lee, 2012a) concluded that lipids are proficient at inducing both autophagy and mTOR signaling and that rapamycin treatment can reduce bacterial viability. Rapamycin is an immunosuppressant drug which prevents the activation of T – and B cells by inhibiting interleukin-2 production (Gonzalez et al., 2001). In addition, starvation induced autophagy is one mechanism by which mycobacterial survival within macrophages can be restricted (Zullo and Lee, 2012a).

1.2.3.3.2 Evasion of autophagy by intracellular bacteria

A form of autophagy, known as xenophagy, targets invading bacteria (Sakowski et al., 2015). Xenophagy works on the same principle as autophagy except that the cargo that is captured by the autophagosome are intracellular pathogens (Paulus and Xavier, 2015).

As mentioned in section 1.2.3.1, the activation of macrophages by the release of *IFN-γ* is an important element in the immune response against invading *M.tb*. Interestingly, in a study by Gutierrez and colleagues (2004), it was shown that the ability of *IFN-γ* to stimulate mycobacterial clearance within macrophages was dependent on an intact autophagy pathway. These investigators showed that *IFN-γ* was able to stimulate autophagy and that inhibition of autophagy by chemical interventions led to an increase in bacterial load. Autophagosomes have also been shown to export cytosolic proteins to vesicles containing *M.tb* in a process mediated by the adaptor protein, p62 (Ponpuak et al., 2010). These proteins include ubiquitin and the ribosomal protein, rps30, which is cleaved and degraded by lysosomal hydrolases to form potent anti-mycobacterial cationic peptides (Alonso et al., 2007; Ponpuak et al., 2010). The digestion of mycobacteria by these anti-mycobacterial peptides releases antigenic peptides that can be presented on the cell surface via MHC class I and II complexes. The antigens can then be detected by antigen-specific CD4+ and CD8+ T cells (Crotzer and Blum, 2010; Deretic, 2011; Deretic et al., 2015).

The immunogenicity of mycobacterial antigens has been found to be enhanced by treatment with rapamycin and BCG. These results suggests that autophagy may represent an alternative mechanism by which potent, focused immune responses are initiated against intracellular targets such as mycobacteria (Zullo and Lee, 2012a).

If an exogenous stimuli is used to activate autophagy, only a few intracellular *M.tb* bacilli will be eliminated due to this amount being naturally susceptible to basal autophagy. On the other hand, a small amount of intracellular *M.tb* bacilli will escape from conventional phagosomes or interact with the cytosol. Interaction with the cytosol can subject the bacilli to be spontaneously eliminated by xenophagy [autophagic targeting of invasive bacteria

(Kuballa et al., 2012)]. In order to eliminate the majority of the intracellular bacilli which are vulnerable to autophagy elimination, autophagy should be induced by physiologic or immunologic means (Deretic et al., 2015).

1.2.3.3.3 Autophagy, the mammalian target of rapamycin pathway and *Mycobacterium tuberculosis*

Infection by both pathogenic and non-pathogenic mycobacteria results in an increase in the mTOR activity, which indicates that the induction of autophagy by mycobacteria takes place in an mTOR-independent manner. Autophagy induction and mTOR signaling happen simultaneously during mycobacterial infection and the responses elicited by host autophagy with regards to any mycobacterium stem from various factors, for instance the inhibitory mechanisms and the presence of activating macromolecules (Zullo and Lee, 2012a).

1.2.3.3.4 Eis gene

One of the genes that enhances *M.tb's* survival is the "enhanced intracellular survival" (*eis*) gene and its protein product, Eis, and this protein modulates the host innate responses and cell death by using ROS (reactive oxygen species)-dependent pathways (Shin et al., 2010a). The *eis* gene has been shown to give *M. smegmatis* the ability to evade macrophage killing and thus has a significant influence on the pathogens' pathogenicity (Meena and Rajni, 2010). Eis functions to inhibit the proliferation of activated T cells, blocks the phosphorylation of ERK (extracellular signal-regulated kinase), and reduces *TNF-α* and *IL-4* (interleukin 4) production. When *eis* is deleted it significantly increases autophagy and inflammatory responses, as well as JNK-dependent generation of ROS in macrophages (Shin et al., 2010a; Kuballa et al., 2012). Cytokines such as *TNF-α* has been shown to activate autophagy and thus it is possible that *M.tb's* cell components can increase the activation of autophagy through inducing the production of *TNF-α*. An *eis* promoter mutation further increases the expression of *eis* and this expression conferred resistance to a first-line antibiotic, kanamycin, in *M.tb* clinical strains, through increasing the acetylation and inactivation (Shin et al., 2010a).

1.2.4 TB host genetics

Although one third of the world population is infected with *M. tuberculosis* (Seto et al., 2012), only 5-10% of these individuals will progress to having active disease while the rest remain asymptomatic (Cliff et al., 2015). This outcome is determined by a number of factors, which include the environment of the host (access to health care, malnutrition, co-infection with other pathogens), the *M. tuberculosis* strain and by the individuals' immune response to the pathogen, which is essentially governed by the genetic makeup of the host (Berry et al., 2013).

The role of host genetic factors with regards to the outcome of the infection and disease has been studied through twin studies, adoption studies, genome-wide linkage and population-based case-control association studies (Patarčić et al., 2015). An indication that a genetic component was involved in TB susceptibility was shown in the outcomes of the Lübeck catastrophe in 1930 in which 252 new born infants were accidentally immunized with the same dose of a virulent TB strain instead of the vaccine strain ("THE LÜBECK CATASTROPHE," 1931; Rieder, 2003; Kaufmann, 2006) were investigated. Of the 252 infected infants, 67 died due to TB; five died from other causes; 108 were still alive but very ill; and 72 infants were seemingly healthy ("THE LÜBECK CATASTROPHE," 1931; Rieder, 2003; Kaufmann, 2006). With regards to gender, the male infants were much more at risk where 27% died compared to 15% female infants ("THE LÜBECK CATASTROPHE," 1931; Rieder, 2003; Kaufmann, 2006).

The role of the host genome was illustrated in the 1890s where the inhabitants of the Qu'Appelle Indian Reservation in Canada were exposed to TB. Of the initially exposed individuals 10% died per year and this was due to a shortage of innate resistance through the absence of prior exposure to the bacterium. More than half the families had died after 40 years; thereafter the death rate had lowered to 0.2%. This decline was attributed to the strong selection against susceptibility genes for tuberculosis (Ferguson, 1934; Möller et al., 2010).

Twin studies are most commonly used to assess heritability where monozygotic twins (genetically identical) are compared to dizygotic twins (approximately 50% identical) in order to define the concordance for the specific condition of interest (Comstock, 1978). A genetic influence on mortality was confirmed by a twin study conducted with information from Denmark, Sweden and Finland (Petersen et al., 2002). The backgrounds of exposure to *M.tb* together with social and environmental conditions were taken into account when making conclusions, and the necessity of these factors to be similar or even identical were declared. In a review article by Meyer and Thye (2014) they mentioned that the concordance of the *M.tb* pathogen was 66% in monozygotic twins and a substantially lower value of 23% was found in dizygotic twins. Although the earlier studies did not follow stringent requirements as the current studies do, the information obtained is still valuable and these studies identified that TB is to some extent a “genetic” disease. This idea was later confirmed through observational differences in TB susceptibility in different population groups (Meyer and Thye, 2014).

Twin and adoption studies are used to separate environmental and genetic effects and to determine their strengths on diseases (Petersen et al., 2002). Sørensen et al. (1988) studied the genetic influence on premature death in adults adopted at an early age and found that death due to infections are strongly related to their genetic background, whereas cardiovascular disease and cancers are related to the family environment. According to Spicer and colleagues (2015) international adoptees are significantly more at risk for TB infection, due to prior living conditions that can include crowed orphanages, malnourishment and birth countries with a prevalence for TB. In a Danish adoption study it was found that premature death in adults were attributed to genetic background and that familial environmental influences did not play much of a role (Petersen et al., 2002). Heritability analysis is a technique used in population genetics to determine what influence genetics has on a specific phenotype (Möller et al., 2010). Heritability studies have estimated TB susceptibility to range between 36% and 80% (Jepson et al., 2001; Newport et al., 2004).

In order to find genetic loci that contribute to TB susceptibility, a number of different approaches have been used. One of these approaches is linkage studies, and should ideally indicate major genes that cause susceptibility in a disease, but it has not been the case thus far (Burgner et al., 2006). One genome-wide linkage scan identified the very first genetic resistance factor for TB infection. This study was made up of 128 families from South Africa, which includes 350 siblings, and surprisingly 40% had no reaction to the TST which was much higher than expected. Two discoveries were made from the linkage analysis, namely that a single locus on chromosomal region 11p14 is responsible for controlling human resistance to the tubercle and a locus on chromosome 5p15 is responsible for the intensity of the TST response (Cobat et al., 2009).

Candidate gene association studies are most commonly used in the investigation of TB susceptibility. There are two possible designs to choose from, namely population-based case-control design or a family-based design (Laird & Lange, 2006; Möller et al., 2010). The first design focuses on the relationship between genetic markers and TB by comparing genotype frequencies from patients who are unrelated and controls, while the second design uses pedigrees and determines the transmission of a marker allele from the heterozygous parents to their affected children by making use of the transmission disequilibrium test (TDT) (Laird & Lange, 2006; Möller et al., 2010). Appendix 1 contains a table with the results of association studies which investigated TB susceptibility candidate genes, which was adopted from Möller et al., (2010).

Tuberculosis is known to be influenced by multiple genetic, environmental and bacterial factors, and therefore it is likely the outcome of *M.tb* infection could be determined by gene interactions (Möller et al., 2010). A multilocus and multigene approach is suitable to analyse the gene-gene interactions of a complex disease such as TB (Möller et al., 2010).

1.3 THE PRESENT STUDY

An association between the immunity related GTPase M *(IGRM)* gene which encodes an autophagy-modulating factor and increased susceptibility to TB was found in a number of

independent human genetic studies (Intemann et al., 2009; Che et al., 2010; King et al., 2011; Bahari et al., 2012). Mice that lack the expression of the *IRGM* orthologue, *LRG47*, were unable to control the *M.tb* and died shortly after infection (MacMicking et al., 2003). Additionally, polymorphisms found in the gene encoding the vitamin D receptor was found to be associated with TB susceptibility when combined with low calcitriol concentrations (the precursor to the active form of vitamin D) (Wilkinson et al., 2000). Calcitriol has recently been functionally linked to the autophagic elimination of *M.tb* (Yuk et al., 2009; Shin et al., 2010a; Campbell and Spector, 2012).

The requirement for autophagy in the host cell response to *M.tb* infection was highlighted by the fact that virulent mycobacteria have evolved specific mechanisms to inhibit autophagy (Kumar et al., 2010). Various studies has indicated that the bacterium has the ability to proliferate within an infected macrophage through inhibiting the fusion of autophagosomes to lysosomes (Armstrong and Hart, 1971). In 2010 the *M.tb* eis gene (section 1.1.4.7) was shown to regulate the host autophagic response (Shin et al., 2010b) and a study by Seto and co-workers (2012) revealed that *M.tb* corrupts the function of the host's coronin 1a protein. This protein localises to phagosomes containing *M.tb* and allows for the survival of the bacteria within macrophages (Seto et al., 2012). It has also been reported that the action of isoniazid and pyrazinamide (section 1.1.3.2) require the host autophagic response to ensure their effectiveness against intracellular mycobacteria (Kim et al., 2012).

The present study will investigate the induction of autophagy in THP-1 cells following infection with seven different *M.tb* strains by examining the differential gene expression of 84 autophagy-related genes. Following this, the top 30 differentially expressed genes will be used as candidate genes in a case-control association study in a cohort of South African Coloured TB cases and control individuals to determine whether they are associated with increased TB susceptibility.

The present study forms part of a larger study investigating the induction of autophagy by different *M.tb* strains using several different approaches. In the larger study, conventional

methods of investigating autophagy induction and protein degradation, such as detection of LC3 using Western blots, fluorescence microscopy and co-localization of lysosomes were used. In the present study, gene expression analysis was used as a means investigate autophagy capacity as well as to identify novel TB susceptibility genes."

1.4 HYPOTHESIS

Since there are interspecies differences in mycobacteria with respect to induction and magnitude of the autophagic response, we hypothesize that this may also be the case within species, contributing to the understanding of the observed differences in virulence between *M.tb* strains. Additionally, we hypothesize that autophagy associated genes that are up-or down-regulated in response to infection with *M.tb*, are good candidate TB susceptibility genes.

1.5 AIMS AND OBJECTIVES

Aims of the study

- Compare gene expression profiles of host autophagy-related genes induced by different strains of *M.tb.*
- To evaluate differentially expressed genes identified in aim 1 as possible candidate genes for increased susceptibility to TB.

Objectives of the study

- To compare gene expression profiles of host autophagy-related genes induced by different strains of *M.tb*.
- To assess known single nucleotide polymorphisms (SNPs) in genes prioritised from gene expression profiles for association with TB susceptibility.

1.6 ETHICAL CONSIDERATIONS

All experiments using virulent strains of *M.tb* were conducted in a biohazard level three (BL-3) laboratory according to standard operating procedures.

The genetic case-control association analysis of this study forms part of a larger study investigating the role of genetic factors involved in TB susceptibility (N95/072). Written informed consent was obtained from all study participants.

Chapter Two

Materials and Methods

CHAPTER TWO

MATERIALS AND METHODS

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MATERIALS AND METHODS

2.1. MYCOBACTERIAL STRAINS AND CELL LINES

2.1.1. Mycobacterial strains

Seven *M.tb* strains representing six *M.tb* lineages were selected for the present study (fig 2.1). All of the strains used in the present study were clinical isolates isolated from TB patients from two suburbs in Cape Town with a high incidence of TB. The strains were collected as part of a large epidemiological study conducted by Prof Rob Warren from the Division of Molecular Biology and Human Genetics, Stellenbosch University. The selected clinical isolates (strains) and the lineage they belong to can be seen in table 2.1. All work conducted using these *M.tb* clinical isolates were done in a biosafety level three (BSL3) laboratory following standard operating procedures.

Table 2.1. The selected clinical isolates (strains) with their corresponding lineages.

Isolate number	**Lineage**
SAWC 3740	CAS/Kili
SAWC 6519	Atypical Beijing
SAWC 1116	Typical Beijing
SAWC 2336	Low Copy Clade (LCC), Four Bander
SAWC 6680	Haarlem 3 (Family 4)
SAWC 2511	LAM 1 (Family 3)
H37Rv	Laboratory Strain

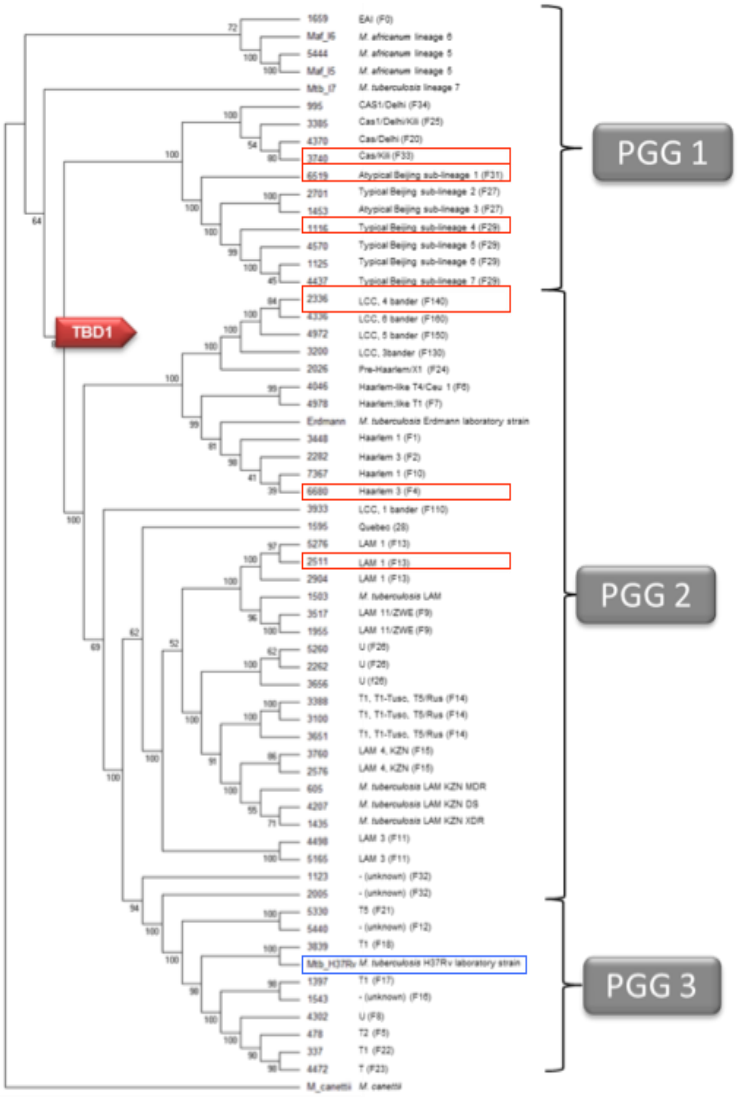

Figure 2.1. Phylogenetic tree of *M.tb* with the strains of interest indicated. The phylogenetic tree is broadly divided into two sections based on the presence or absence of TbD1, a specific *M.tb* deletion. The presence of TbD1 indicates the strain is ancient, compared to a modern strain where TbD1 is absent (Brosch et al., 2002a). PGG = Principle Genetic Group (Gutacker et al., 2006).

2.1.1.1 Mycobacterial Culture

One milliliter (mL) of *M.tb* (stock) was re-suspended in 20mL Middlebrook 7H9 liquid medium (appendix 2) in a T75 CELLSTAR® Cell Culture Flask (Greiner Bio-One, Frickenhausen, Germany). The culture incubated horizontally at 37°C in an incubator for 5 to 14 days. The optical density (OD) at 600nm was measured in an Ultrospec 4051, LKB Biochrom Ltd. (Biochrom Ltd., Cambridge, United Kingdom), and every 5 days. The bacteria was aliquoted into 1mL screw cap tubes (Sigma-Aldrich® Co, Missouri, USA) once the OD reached 0.800 – 1.000. One mL of the culture was used for determining the amount of viable *M.tb* colonies and is described in 2.1.1.2.

2.1.1.2 Determination of colony forming units

Serial dilutions (from 10^{-1} to 10^{-7} in duplicate) of the each of the *M.tb* stocks (section 2.1.1.1) were prepared for determination of colony forming units (CFUs). One hundred microliters (µL) of each dilution was plated onto Middlebrook 7H11 agar plates which were subsequently incubated at 37℃ for approximately two weeks for *M.tb* strains or three days for *M.smeg*. Following this, the bacterial colonies on the plates were counted and CFUs per milliliter were calculated as follow: the number of colonies on the plate was counted and divided by the dilution factor (normally 10^{-6} or 10^{-7}) and the volume (mL) which was plated from the stock. Thus the concentration is then used to determine the volume of bacteria needed to do a successful infection (section 2.2.2).

$$Concentration\left(\frac{CFU}{mL}\right) = \frac{Average\ CFU\ count}{volume\ (mL) x\ dilution\ factor}$$

2.1.2 THP-1 cells

THP-1 is a human leucemia cell line derived from a patient with acute monocytic leucemia. THP-1 cells are monocytes and therefore it is necessary to differentiate it into macrophages in order to use it in our study. THP-1 cells are round and grow in grape-like clusters in suspension, and prefer to grow in a single layer. THP-1 cells have a doubling time of 35 – 50 hours and this makes these cells 'slow-growers' (Tsuchiya et al., 1980).

2.1.2.1 Thawing of cells

The THP-1 cells in the cryotubes were defrosted in a 37°C BH-130 water bath (Yihdern Co., Ltd, Xinbei City) and subsequently aliquoted into 12mL Greiner tubes (Greiner Bio-One, Frickenhausen, Germany). Following this, 4mL of RPMI-1640 media (Lonza BioWhittaker®, Cape Town, South Africa) supplemented with 10% FBS (50mL) and 0.01% L-glutamine (5mL) was added to the cells. The tubes were centrifuged for four min at 1400rpm (revolutions per minute) using a Z206A centrifuge (Lasec, Cape Town, South Africa), and the media was subsequently discarded. Thereafter the cells were re-suspended in 6-10mL (depending on the amount of cells) pre-warmed RPMI-1640 supplemented media. The cells were transferred to a T25 CELLSTAR® Cell Culture Flask (Greiner Bio-One, Frickenhausen, Germany) and incubated at 37°C in a Farma termosteri-cycle 5% carbon dioxide humidified incubator (Farma International, Miami, Florida, USA). The growth was checked daily.

2.1.2.2 Passaging cells (splitting the cells)

The media containing the cells from the T25 CELLSTAR® Cell Culture Flask (Greiner Bio-One, Frickenhausen, Germany) was transferred to a 12mL Greiner tubes (Greiner Bio-One, Frickenhausen, Germany). The cells were centrifuged for 4min at 1400rpm in a Z206A centrifuge (Lasec, SA). The media was slowly decanted until only 2mL was left. Thereafter 6-10mL of pre-warmed RPMI-1640 supplemented media was added to the 12mL tube. The contents of the tube were divided between two new T25 CELLSTAR® flasks. More RPMI-1640 supplemented media was added when necessary. Once again the T25 CELLSTAR® flasks were incubated at 37°C with 5% CO_2 and the daily growth checked.

2.1.2.3 Storing cells in liquid nitrogen

Once the cells reached an 80% confluency they were either passaged or stored in liquid nitrogen. In order to store the cells, the media containing the THP-1 cells were transferred to a 12mL tube and centrifuged at 3000rpm for three min in a Z206A centrifuge (Lasec, SA) and the pellet was re-suspended in 4mL of RPMI-1640 supplemented media. One milliliter of cells were transferred to each of four cyrotubes followed by 1mL freezing media

(appendix 2). The cells were stored at -80℃ overnight and the following day, the tubes were transferred into liquid nitrogen storage.

2.2. INFECTION OF THP-1 CELLS WITH MYCOBACTERIAL STRAINS

2.2.1 Haemocytometric cell count and seeding of THP-1 cells

Cell count was determined using a Neubauer haemocytometer (Superior, Berlin, Germany) in order to calculate the concentration of the THP-1 cells used when performing infections with *M.tb*. Prior to counting, both the surface of the chamber and the glass cover-slip was cleaned with alcohol. The glass cover slip was then placed over the counting surface (fig 2.2); a dilution of (1:100) of the THP-1 cells prepared an aliquot of 10µL was placed into the V-shaped wells. Once the area under the coverslip filled with the sample through capillary action, the counting chamber was subsequently placed on a microscope (Nikon Corporation, Tokyo, Japan) stage and the counting area was brought into focus under low magnification. The number of cells in the large central quadrant of the haemocytometer (fig 2.3) were counted and this value was used to calculate the number of cells per mL using the following formula: Number of cells/mL – number of cells x dilution factor x 10^4 (a constant used due to the depth (0.1mm) of the haemocytometer).

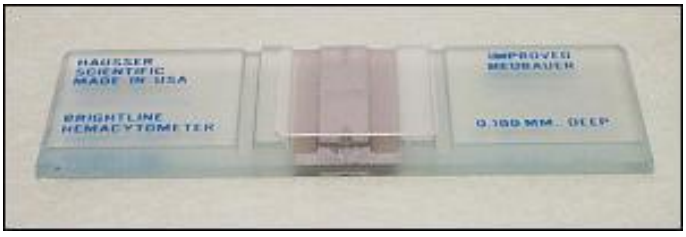

Figure 2.2. Coverslip on a haemocytometer.

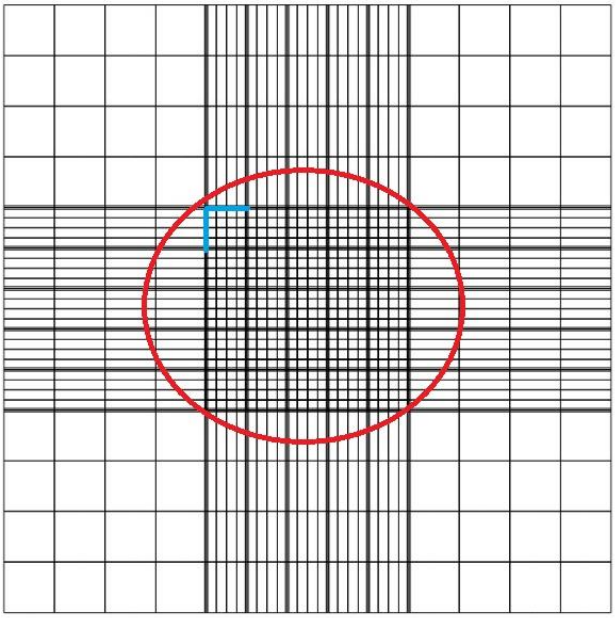

Figure 2.3. Representation of haemocytometer squares under the microscope. Focus on the squares indicated by the red circle and only count the cells inside each block or if they are on the top or left border (indicated in blue).

2.2.2. Differentiation of THP-1 cells

Approximately 50 000 THP-1 cells were seeded in 1mL of RPMI-1640 supplemented with 10% FBS and 1% Pen/Strep in each well of a 24-well cell culture plate. Following this, 1mL of 100µg/mL Phorbol 12-Myristate 13-Acetate (PMA) was added to each well in order to differentiate the THP-1 cells to macrophages. The cells were then incubated at 37°C / 5% CO_2 for three days in a Farma termosteri-cycle 5% carbon dioxide humidified incubator (Farma International, Miami, Florida, USA). After the three days the media was removed, fresh media was added and the cells were incubated for an additional day. After the addition or removal of media from the 24-well plate, the cells were observed under a microscope to determine if the cells were still viable.

2.2.3 Infection of the THP-1 cells with *M.tb*

For all infections, a multiplicity of infection (MOI) of five was used. The MOI is the ratio of bacteria to cells, thus an MOI of 5 means that, on average, each cell was infected with five mycobacterial bacilli. Keane et al. (1997) were the first to report that a MOI of 5 was

adequate to induce cell death. Due to strains with different virulence and growth rates, a MOI of 5 was ideal because we did not want cell death to occur before we could extract the RNA and we did not want to affect the *M.tb's* viability by using a high MOI (Lee et al., 2009).

The vials containing the different bacteria strains (section 2.1.1.1) were thawed and the calculated amount of bacteria was added to clean RPMI-1640 media in clean tubes. To ensure the bacteria were not clumped, the culture was passed through a 1mL/cc insulin syringe (Supra Latex, Cape Town, S.A.) 15 times without making bubbles.

THP-1 cells were seeded as described in section 2.2.1. The media was removed from all the wells and the appropriate amount *M.tb* (MOI=5) diluted in the calculated amount of RPMI-1640 was added to triplicate wells. The cells were incubated at 37°C in a Farma termosteri-cycle 5% carbon dioxide humidified incubator (Farma International, Miami, Florida, USA) for 4 hours to ensure infection occurred. After 4 hours, the diluted bacteria was removed from each well, the cells were washed with clean RPMI-1640 media. Following this, 1mL of RPMI-1640 supplemented with 1% Pen/Strep was added to each well and incubated for another hour. After the additional hour the RPMI-1640 supplemented media treated with Pen/Strep was removed and clean RPMI-1640 media was added.

2.4. GENE EXPRESSION ANALYSIS

2.4.1. RNA extraction

In order to isolate RNA from THP-1 cells cultured in 24-well plates, the Qiagen RNeasy® Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's protocol. Briefly, the culture media was completely removed from each well and 350µL Buffer RTL was added to each well of the 24–well plate. The lysate was collected with a rubber policeman, transferred to a 1.5mL microcentrifuge tube and mixed by pipetting. The lysate was then pipetted into a QIAshredder spin column (Qiagen, Hilden, Germany) placed in a 2mL collection tube, and centrifuged for 2 min at 15000rpm in a Breeze NU-C2500R

Refrigerated Laboratory microcentrifuge (Lasec, SA). The steps described thus far have been carried out in the BSL-3 laboratory following standard procedures.

Before the flow-through collected in the 2mL collection tube can be brought out of the BSL-3 laboratory, the QIAshredder tube has to be disposed of and the collection tube wiped clean with distel disinfectant. Once outside of the BSL-3 laboratory the protocol was followed without any interruptions. One volume of 70% ethanol was added to the homogenised lysate and mixed by pipetting. The sample was transferred to an RNeasy spin column placed in a 2mL collection tube. The flow-through was discarded after the tube was centrifuged for 15 sec at 10 000rpm. Seven hundred microliters buffer RW1 was added to the RNeasy spin column and centrifuged for 15 sec at 10 000rpm to wash the spin column membrane. Five hundred microliters Buffer RPE was then added to the RNeasy spin column, centrifuged for 15 sec at 10 000rpm and the flow-through was discarded. The column was then centrifuged for 2 min at 10 000rpm to wash the spin column membrane after 500µL of buffer RPE was added. The RNeasy spin column was then placed in a new 2mL collection tube and centrifuged at full speed for 1 minute, while the used collection tube with the flow-through is discarded. Once centrifuged, the RNeasy spin column was placed in a new 1.5mL collection tube and 20µL RNase-free water was added directly to the spin column membrane. The tube was centrifuged for an additional 1 minute at 10 000rpm in order for the RNA to be eluted and the RNase-free water step was repeated. After RNA extraction was completed, the quality and concentration of RNA was determined using the Agilent 2100 BioAnalyser (Agilent Technologies, Santa Clara, USA).

2.4.2 Determination of RNA concentration and integrity

RNA concentration and integrity was determined at the Central Analytical Facility, Stellenbosch University using the Agilent RNA 6000 nanokit and Agilent 2100 BioAnalyser (Agilent Technologies, Santa Clara, USA). Briefly, the RNA samples were heat denatured at 70°C for 2 min before loading them onto the RNA nanochip. The RNA ladder (1µL) was pipetted into the ladder well and 1µL of each sample was pipetted into the rest of the wells. The chip was placed horizontally in the adapter of the IKA vortex

mixer and vortexed at 2 000rpm for 60 sec. The chip was subsequently placed into the Agilent 2100 BioAnalyser for virtual gel electrophoresis. The data was analysed using the 2100 Expert Software.

2.4.3 cDNA conversion

In order to reverse transcribe the isolated RNA (section 2.4.1) into complementary DNA (cDNA), the Qiagen RT^2 first strand kit (Qiagen, Hilden, Germany) was used according to the manufacturer's protocol. Genomic DNA elimination mix was prepared by combining 2µL of GE buffer with 100ng of sample RNA and RNase-free water to a total volume of 14µL and incubated at 42ºC for 5 min. Following incubation the genomic DNA elimination mix was immediately placed on ice of 2 min. Reverse transcription master mix was prepared for each RNA sample as follows: buffer BC3 at a final concentration of 2.5X, 1µL of the P2 control, 2µL of the RE3 Reverse Transcriptase Mix and RNase-free water to a final volume of 10µL per sample. 10µL of the reverse transcription mix was added to each genomic elimination mix sample and incubated at 42ºC for 15 min. Tubes were subsequently transferred to a heating block and incubated at 95ºC for 5 min to stop the reaction. Finally 91µL of RNase-free water was added to each sample and mixed by pipetting action. The cDNA was subsequently stored at -80ºC until needed.

2.4.4 Real-time quantitative PCR

Differential gene expression of 84 autophagy-related genes (table 2.2) were determined using RT^2 Profiler™ predesigned autophagy qPCR Human Array System from SA BioSciences™ (Eppendorf, Hamburg, Germany). These pre-designed arrays contain SYBR Green-optimized primer assays for 84 genes that have been shown to be involved in autophagy as well as five housekeeping genes for normalization of the expression data and to determine whether the extracted RNA samples were contaminated with genomic DNA. Furthermore, positive PCR controls were also present on each array (figure 2.4).

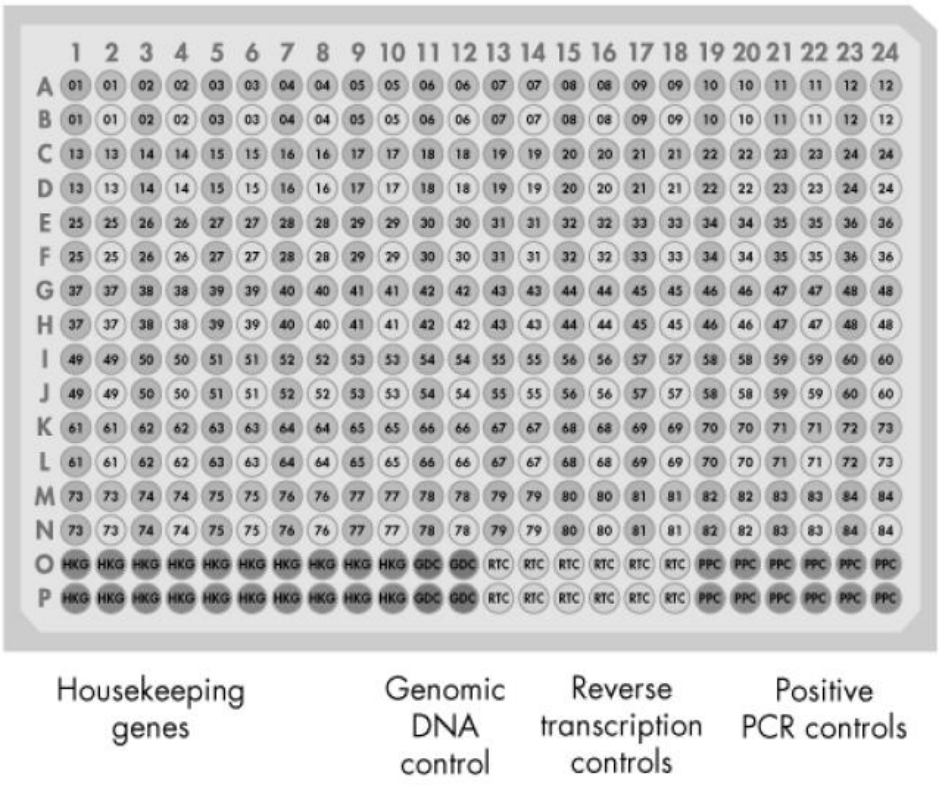

Figure 2.4. A schematic representation of how the design of the plate was determined. Samples were run in duplicate with two samples per plate.

Two cDNA samples (from different infections) were PCR-amplified in biological duplicates on each array. A PCR-mastermix for each sample was prepared as follows: In a 1.5mL microfuge tube, 650µL of 2x RT^2 SYBR Green Mastermix was added to 102µL cDNA (section 2.4.3) and 548µL RNAse-free water. Following this, 10µL of each sample was added to appropriate wells of the array.

Real-time PCR was performed using the 7900HT Fast Real-Time PCR instrument. Cycling conditions were as follows: 95ºC for 1 min to allow for HotStart Taq Polymerase activation, followed by 40 cycles of 95ºC for 15 sec and 60ºC for 1 min at which point fluorescence data capture was done.

2.4.5. RT2 Expression Profile Analysis

Gene expression data was analysed using the RT^2 Profiler PCR Array Data Analysis version 3.5 software package available at the integrated web-based software package available at http://www.sabiosciences.com/pcrarraydataanalysis.php. The software makes use of the 2-ΔΔCt method using all five of the included housekeeping genes in the analyses to provide fold-changes of genes in each infection group versus the uninfected control group. The software also analyses the internal genomic, reverse transcriptase and positive amplification controls for quality assessment.

Table 2.2. The 84 autophagy-related genes that are coated on the RT^2 Profiler predesigned autophagy qPCR Array.

Position	UniGene	GenBank	Symbol	Description
A01	Hs.525622	NM 005163	*AKT1*	V-akt murine thymoma viral oncogene homolog 1
A02	Hs.654644	NM 017749	*AMBRA1*	Autophagy/beclin-1 regulator 1
A03	Hs.434980	NM 000484	*APP*	Amyloid beta (A4) precursor protein
A04	Hs.713698	NM 031482	*ATG10*	ATG1O autophagy-related 1O homolog (S. cerevisiae)
A05	Hs.264482	NM 004707	*ATG12*	ATG12 autophagy-related 12 homolog (S. cerevisiae)
A06	Hs.529322	NM 017974	*ATG16L1*	ATG 16 autophagy-related 16-like l (S. cerevisiae)
A07	Hs.653186	NM 033388	*ATG16L2*	ATG16 autophagy-related 16-like 2 (S. cerevisiae)
A08	Hs.477126	NM 022488	*ATG3*	ATG3 autophagy-related 3 homolog (S. cerevisiae)
A09	Hs.8763	NM 052936	*ATG4A*	ATG4 autophagy-related 4 homolog A (S. cerevisiae)

Position	UniGene	GenBank	Symbol	Description
A10	Hs.283610	NM 178326	*ATG4B*	ATG4 autophagy-related 4 homolog B (S. cerevisiae)
A11	Hs.7353	NM 178221	*ATG4C*	ATG4 autophagy-related 4 homolog C (S. cerevisiae)
A12	Hs.512799	NM 032885	*ATG4D*	ATG4 autophagy-related 4 homolog D (S. cerevisiae)
B01	Hs.486063	NM 004849	*ATG5*	ATGS autophagy-related 5 homolog (S. cerevisiae)
B02	Hs.740389	NM_006395	*ATG7*	ATG7 autophagy-related 7 homolog (S. cerevisiae)
B03	Hs.323363	NM 024085	*ATG9A*	ATG9 autophagy-related 9 homolog A (S. cerevisiae)
B04	Hs.707300	NM 173681	*ATG9B*	ATG9 autophagy-related 9 homolog B (S. cerevisiae)
B05	Hs.370254	NM 004322	*BAD*	BCL2-associated agonist of cell death
B06	Hs.485139	NM 001188	*BAK1*	BCL2-antagonist/killer l
B07	Hs.624291	NM 004324	*BAX*	BCL2-associated X protein
B08	Hs.150749	NM 000633	*BCL2*	8-cell Cll/lymphoma 2
B09	Hs.516966	NM 138578	*BCL2L1*	BCL2-like 1
B10	Hs.716464	NM 003766	*BECN1*	Beclin 1, autophagy-related
B11	Hs.517145	NM 001196	*BID*	BH3 interading domain death agonist
B12	Hs.144873	NM 004052	*BNIBSL-3*	BCL2/adenovirus ElB l9kDa interading protein 3
C01	Hs.141125	NM 004346	*CASBSL-3*	Caspase 3, apoptosis-related cysteine peptidase

Position	UniGene	GenBank	Symbol	Description
C02	Hs.599762	NM 001228	*CASP8*	Caspase 8, apoptosis-related cysteine peptidase
C03	Hs.238990	NM 004064	*CDKN1B*	Cyclin-dependent kinase inhibitor lB (p27, Kipl)
C04	Hs.512599	NM 000077	*CDKN2A*	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)
C05	Hs.534667	NM 000086	*CLN3*	Ceroid-lipofuscinosis, neuronal 3
C06	Hs.520898	NM 001908	*CTSB*	Cathepsin B
C07	Hs.654447	NM 001909	*CTSD*	Cathepsin D
C08	Hs.181301	NM 004079	*CTSS*	Cathepsin S
C09	Hs.593413	NM 003467	*CXCR4*	Chemokine (C-X-C motif) receptor 4
C10	Hs.380277	NM 004938	*DAPK1*	Death-associated protein kinase 1
C11	Hs.525634	NM 018370	*DRAML*	DNA-damage regulated autophagy modulator 1
C12	Hs.485606	NM 178454	*DRAM2*	DNA-damage regulated autophagy modulator 2
D01	Hs.591589	NM 004836	*EIF2AK3*	Eukaryotic translation initiation factor 2-alpha
D02	Hs.433750	NM 182917	*EIF4G1*	Eukaryotic translation initiation factor 4 gamma, 1
D03	Hs.744830	NM 000125	*ESR1*	Estrogen receptor 1
D04	Hs.86131	NM 003824	*FADD*	Fas (TNFRSF6)-associated via death domain

Position	UniGene	GenBank	Symbol	Description
D05	Hs.667309	NM 000043	*FAS*	Fas (TNF receptor superfamily, member 6)
D06	Hs.1437	NM 000152	*GAA*	Glucosidase, alpha; acid
D07	Hs.647421	NM 007278	*GABARAP*	GABA(A) receptor-associated protein
D08	Hs.524250	NM 031412	*GABARAPL1*	GABA(A) receptor-associated protein like 1
D09	Hs.461379	NM 007285	*GABARAPL2*	GABA(A) receptor-associated protein-like 2
D10	Hs.88556	NM 004964	*HDAC1*	Histone deacetylase 1
D11	Hs.6764	NM 006044	*HDAC6*	Histone deacetylase 6
D12	Hs.661056	NM 004712	*HGS*	Hepatocyte growth factor-regulated tyrosine kinase substrate
E01	Hs.525600	NM 001017963	*HSP90AA1*	Heat shock protein 90kDa alpha (cytosolic), class A
E02	Hs.180414	NM 006597	*HSPA8*	Heat shock 70kDa protein
E03	Hs.518450	NM 002111	*HTT*	Huntingtin
E04	Hs.856	NM 000619	*IFNG*	Interferon, gamma
E05	Hs.160562	NM 000618	*IGF1*	Insulin-like growth factor 1 (somatomedin C)
E06	Hs.700350	NM 000207	*INS*	Insulin
E07	Hs.519680	NM 001145805	*IRGM*	Immunity-related GTPase family, M
E08	Hs.494419	NM 005561	*LAMP1*	Lysosomal-associated membrane protein 1
E09	Hs.632273	NM 181509	*MAP1LC3A*	Microtubule-associated protein 1 light chain 3
E10	Hs.356061	NM 022818	*MAP1LC3B*	Microtubule-associated protein 1 light chain 3
E11	Hs.485233	NM 001315	*MAPK14*	Mitogen-activated protein
E12	Hs.138211	NM 002750	*MAPK8*	Mitogen-activated protein

Position	UniGene	GenBank	Symbol	Description
F01	Hs.338207	NM 004958	*mTOR*	Mechanistic target of rapamycin (serine/threonine kinase)
F02	Hs.618430	NM 003998	*NFKB1*	Nuclear factor of kappa light polypeptide gene
F03	Hs.464779	NM 000271	*NPC1*	Niemann-Pick disease, type C1
F04	Hs.656958	NM 002647	*PIK3C3*	Phosphoinositide-3-kinase, class 3
F05	Hs.32942	NM 002649	*PIK3CG*	Phosphoinositide-3-kinase, catalytic, gamma polypeptide
F06	Hs.149032	NM 014602	*PIK3R4*	Phosphoinositide-3-kinase, regulatory subunit 4
F07	Hs.43322	NM 006251	*PRKAA1*	Protein kinase, AMP-activated, alpha 1 catalytic subunit
F08	Hs.729457	NM 000314	*PTEN*	Phosphatase and tensin
F09	Hs.16258	NM 130781	*RAB24*	RAB24, member RAS oncogene family
F10	Hs.408528	NM 000321	*RB1*	Retinoblastoma 1
F11	Hs.422336	NM 005873	*RGS19*	Regulator of G-protein signaling 19
F12	Hs.463642	NM 003161	*RPS6KB 1*	Ribosomal protein S6 kinase, 70kDa, polypeptide 1

Position	UniGene	GenBank	Symbol	Description
G01	Hs.21374	NM 000345	*SNCA*	Synuclein, alpha (non A4 component of amyloid precursor)
G02	Hs.587290	NM 003900	*SQSTML*	Sequestosome 1
G03	Hs.645227	NM 000660	*TGFB1*	Transforming growth
G04	Hs.517033	NM_004613	*TGM2*	Transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)
G05	Hs.99439	NM 153015	*TMEM74*	Transmembrane protein
G06	Hs.241570	NM 000594	*TNF*	Tumor necrosis factor
G07	Hs.478275	NM 003810	*TNFSF10*	Tumor necrosis factor (ligand) superfamily, member 10
G08	Hs.437460	NM 000546	*TP53*	Tumor protein p53
G09	Hs.47061	NM 003565	*ULK1*	Unc-51-like kinase 1 (C. elegans)
G10	Hs.168762	NM 014683	*ULK2*	Unc-51-like kinase 2 (C. elegans)
G11	hs.202470	NN1 003369	*UVRAG*	UV radiation resistance
G12	Hs.463964	NM 017983	*WIPI1*	WD repeat domain, phosphoinositide interacting 1
H01	Hs.520640	NM 001101	*ACTB*	Actin, beta
H02	Hs.534255	NM 004048	*82M*	Beta-2-microglobulin
H03	Hs.592355	NM 002046	*GAPDH*	Glyceraldehyde-3-phosphate dehydrogenase

Position	UniGene	GenBank	Symbol	Description
H04	Hs.412707	NM 000194	*HPRT1*	Hypoxanthine phosphoribosyltransferase 1
H05	Hs.546285	NM 001002	*RPLPO*	Ribosomal protein, large,
H06	N/A	SA 00105	*HGDC*	Human Genomic DNA
H07	N/A	SA 00104	*RTC*	Reverse Transcription
H08	N/A	SA 00104	*RTC*	Reverse Transcription
H09	N/A	SA 00104	*RTC*	Reverse Transcription
H10	N/A	SA 00103	*PPC*	Positive PCR Control
H11	N/A	SA 00103	*PPC*	Positive PCR Control
H12	N/A	SA 00103	*PPC*	Positive PCR Control

2.5 CASE-CONTROL ASSOCIATION STUDY

2.5.1. Ethics approval

The Health Research Ethics committee of Stellenbosch University approved this study with the ethic number 95/072. All the adult study participates and legal guardians gave written informed consent, while approval was obtained for minor children.

2.5.2 Patient recruitment

Study participants were recruited from the South African Coloured (SAC) population residing in two suburbs of Cape Town with a low HIV incidence rate (Beyer et al., 1996) and high incidence of TB (Munch et al., 2003) at the time of sampling. Whole blood samples were collected from all the participants. All the study participants were unrelated to each other and HIV negative. TB diagnoses were done bacteriologically using smear or culture methods for positivity.

The SAC population is an admixed population which is derived from several distinct population groups that settled in the early Cape colony. These include the indigenous Khoisan (32 – 43%), European (21 – 28%), African (20 – 36%) and Asian (Indian) (9 –

11%) populations. The extent to which the SAC population is admixed presents an opportunity to discover possible underlying genetic factors to study multifactorial diseases, such as TB (de Wit et al., 2010).

The healthy controls samples, defined as individuals who had never had a case of active TB in their lifetime, were collected in the same manner as the TB cases and were over the age of 18, unrelated and HIV-negative. A study conducted in this area to determine the impact of sex and age on mycobacterial immunity stated that 80% of the individuals 15 years and older had positive tuberculin skin tests (TST) which is an indication of latent infection. It is therefore likely that the controls (who are 18 and older) were previously exposed to the bacterium and are latently infected (Gallant et al., 2010).

2.5.3 DNA extraction

DNA for this study was extracted prior to this study. In order to extract DNA from whole blood collected from the TB patients and healthy controls participating in the case-control study, the GE Healthcare Nucleon™ BACC2 Genomic DNA Extraction kit (Thermo Fisher Scientific, Massachusetts, USA) was used according to the manufacturer's protocols. The DNA extraction was done as follows (fig 2.5): 400µL of the buffy coat was resuspended in 1mL of solution A (sodium perchlorate), followed by centrifugation at 6000 rpm for 5 min at 4°C. After centrifugation the pellet was resuspended in 700µL of solution A (2 – 3 times). Thereafter the pellet was resuspended in 500µL of solution B and briefly vortexed, 125 µL of sodium perchlorate was then added and the tube was inverted several times. The tube was again inverted after 500µL of chloroform was added and centrifuged at 6500 rpm for 3 min at 4°C after 150µL of Nucleon™ silica was added. The upper phase was transferred to a new tube and 2X cold absolute ethanol was added and centrifuges at 12 000 rpm for 10 min at 4°C. The DNA was washed with 1mL 70% cold ethanol, centrifuged at 12 000 rpm for 10 min at 4°C, left to air dry and then resuspended in TE buffer and subsequently stored at -80ºC until needed.

Figure 2.5. Steps followed for successful extraction of DNA from blood samples using the Nucleon™ BACC2 protocol.

2.5.4 Sample selection for genotyping

As part of a larger study, a total of 1 000 samples were selected and sent for genotyping on the Illumina MEGA® array (Illumina San Diego, CA, USA). We were interested in the 400 pulmonary TB (PTB) cases and 477 healthy controls. DNA quality metrics (260/230 ratio ≤ 2 and 260/280 ≥ 1.7) determined by Nanodrop 2000c and Nanodrop 2000/2000c software (Thermo Fisher Scientific, Massachusetts, USA) were used for selecting PTB and healthy control samples.

The preparation of the samples were done as follows: GE Healthcare Nucleon™ BACC2 Genomic DNA Extraction kit (Thermo Fisher Scientific, Massachusetts, USA) was used for extracting the DNA, samples were diluted to 60ng/µL and aliquoted in 96-well plates; the plates were stored at -20°C during shipping to Hussman Institute for Human Genomics (University of Miami, Florida, USA) where genotyping was performed. All the samples underwent quality control measures once it arrived at the institute to determine DNA quality and quantity. Quality control checks were performed on the DNA before genotyping on the array was performed. Fresh aliquots (17 samples) replaced samples that had significant DNA degradation. After all the quality control procedures were completed, 995 samples and 11 HIHG (Hussman Institute for Human Genomics) were genotyped.

2.5.5 Data Analysis

The top 30 genes with the biggest fold change values as indicated by the RT^2 Profiler (section 4.1.8) were selected for analysis from the MEGA® array data. Plink (version 1.07) (Purcell et al., 2007) was used for analysing the SNPs of interest, which included

the regulatory regions, in the data obtained from the Illumina MEGA® array. In table 2.3 the genes of interest are listed together with their genomic region, where the SNPs are located, that was entered into Plink. All the samples were configured into a table format where the sample ID, relationship, sex, and case/control status was indicated. All the samples in the cohort were unrelated. Next, four files were made which included a .bed file (contained all the genotype information), .fam file (contained the family information), .bin file (contained the SNPs), and .txt file (contained the covariants information). In total there are six covariants which were corrected for, namely the four population groups, age and sex. The population groups were CEU (Utah Residents from North and West Europe), SAN (Upington, South Africa which were given through a collaboration with Dr. Brenna Henn), LWK (Luhya in Webuye, Kenya) and SAS (GIH) (Gujarati Indian from Houston, Texas).

Table 2.3. List of genes that were selected for genotyping with their location that was entered into Plink (v1.07) (Purcell et al., 2007).

Gene	**Location according to the NCBI website** ("Home - Gene - NCBI,")
TNF	31543344..31546113
CDKN2A	21967751..21994490
IFNG	68548550..68553521
RAB24	176728199..176730744
IRGM	150226085..150228231
TMEM74	109791165..109799770
INS	2181009..2182439
LAMP1	113951436..113977746
RGS19	62704534..62711356
MAPK14	35995412..36079013
SQSTM1	179233388..179265078
CTSD	1773982..1785222
TGFB1	41836812..41859831

Gene	**Location according to the NCBI website** ("Home - Gene - NCBI,")
DRAM2	111659954..111682838
CTSS	150702672..150738433
CTSB	11700033..11725646
RPS6KB1	57970407..58027787
MAP1LC3B	87423157..87438380
CLN3	28488600..28505897
HDAC1	32757708..32799227
CDKN1B	12870302..12875305
GAA	78075339..78093680
WIPI1	66417422..66453653
RB1	48877883..49056026
HSP90AA1	102547075..102606086
PRKAA1	40759481..40798297
HGS	79650962..79669151
BID	18216906..18257431
BNIP3	133781204..133795435
GABARAPL2	75600249..75611779

A logistic regression model was constructed per SNP in order to determine whether there was a difference between the null model (that there is an association with SNPs in autophagy related genes and TB susceptibility) and what is observed. Bonferroni correction was used to adjust for multiple testing, while a confidence interval of 95% was selected. After quality control, a total of 771 individuals, comprising 382 PTB cases and 389 controls were included into the study (table. 2.4). 1 061 SNPs were included from the list of genes and a genotyping rate of 99.64% was obtained. In total 755 tests were corrected for. Although multiple testing was corrected, attention was given to nominal associations (values not corrected) based on our experimental evidence that the genes are involved in the autophagy pathway.

Table 2.4. TB cases and controls included in the study.

TB cases		TB control	
Male	212	Male	122
Female	170	Female	267
Total	382	Total	389

Chapter Three

Results

CHAPTER THREE

RESULTS

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Results

3.1 DETERMINATION OF RNA CONCENTRATION AND QUALITY

RNA was quantified and qualified using the Agilent RNA 6000 nanokit and Agilent 2100 BioAnalyser (Agilent Technologies, Santa Clara, USA). An example of the digital RNA images indicating RNA quality can be seen below (Figure 3.1). RNA Integrity Number (RIN) values of 7 or above were considered adequate for subsequent cDNA conversion (Table 3.1).

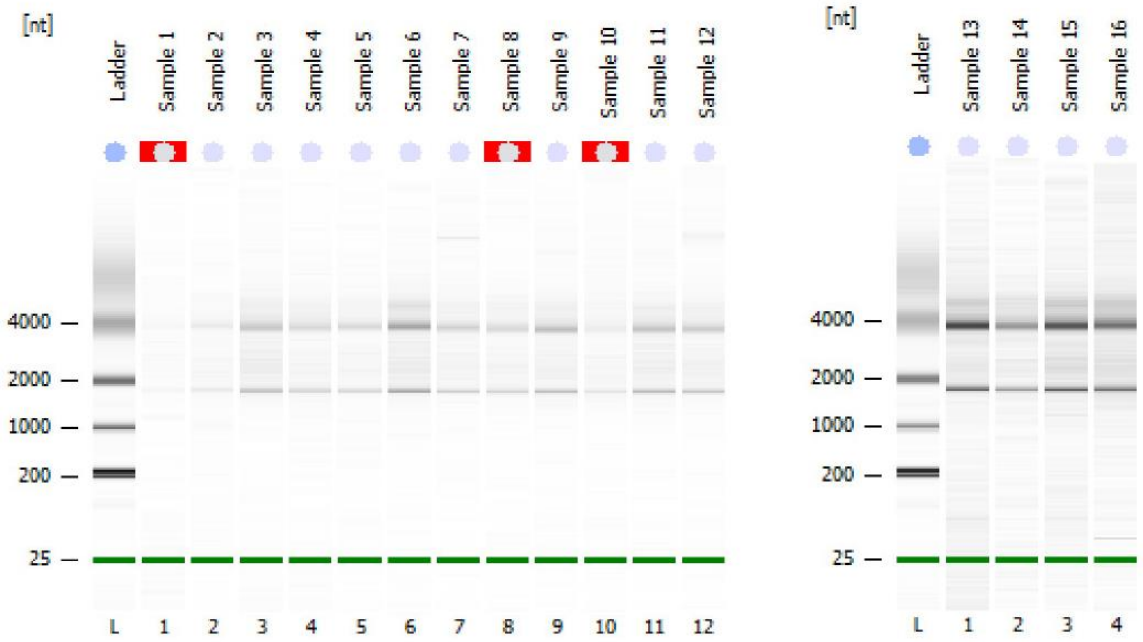

Figure 3.1. Electrophoresis of the 16 samples. The band at 4 000 nucleotides (nt) is 28S and the band at 2 000 nt is 18S. Red blocks indicate samples with RIN values less than 7.

Table 3.1. RIN values for RNA extracted from *M.tb* infected macrophages.

Sample Name	Strain	RIN	Concentration ng/µL
Control A	Uninfected	*N/A*	3
Control B		7.5	12
2511 A	LAM 1 (F13)	9.6	16
2511 B		9	13
H37Rv A	Laboratory strain	9	10
H37Rv B		9.4	22
3740 A	CAS/Kili	9	11
3740 B		*N/A*	10
6519 A	Atypial Beijing sub-lineage 1	9	18
6519 B		*N/A*	7
2336 A	LCC, 4 bander	9.1	17
2336 B		9.5	13
6680 A	Haarlem 3 (F4)	9.7	21
6680 B		8.9	13
1116 A	Typical Beijing sun-lineage 4	8.8	33
1116 B		8.8	26

Abbreviations: CAS, Central Asian Strain; F, Family; LAM, Latin American-Mediterranean; LCC, Low Copy Clade; N/A, Not available (value not reliable for the sample); RIN, RNA Integrity Number

3.2 GENE EXPRESSION ANALYSIS

The RT^2 Profiler PCR Array Data Analysis version 3.5 software package was used to analyse the gene expression data generated by RT-PCR of 84 autophagy associated genes using the RT^2 profiler Autophagy PCR array. The relative gene expression differences of 2-fold or higher between uninfected THP-1 cells and THP-1 cells infected with each of the *M.tb* strains investigated in this study is shown appendix III. In tables 3.2 to 3.10, genes that were up-or-down-regulated 10-fold or more are shown. Since the gene

expression data were used to choose likely candidate genes for TB susceptibility, we chose to investigate genes that were more than 10-fold differentially expressed as these genes are more likely to have a large effect on TB susceptibility. The biological duplicates correlated well and all the samples were included in the analysis, no outliers were identified.

3.2.1 LAM 1 strain

Table 3.2. Up- and down-regulated genes, in THP-1 cell infected with a LAM 1 (F13) *M.tb* strain compared to uninfected cells.

UP- REGULATED (10-fold)		
Array position	**Gene**	**Amount up-regulated**
G06	*TNF*	+27.55
DOWN- REGULATED (10-fold)		
Array position	**Gene**	**Amount down-regulated**
C03	*CDKN1B*	-69.91
E08	*LAMP1*	-41.61
F11	*RGS19*	-28.79
D06	*GAA*	-25.45
E11	*MAPK14*	-23.57
D04	*FADD*	-15.99
B03	*ATG9A*	-15.45
E03	*HTT*	-15.00
A11	*ATG4C*	-14.15
A01	*AKT1*	-12.35
G03	*TGFB1*	-12.31
B09	*BCL2L1*	-12.27
D10	*HDAC1*	-11.82
D12	*HGS*	-11.46
A03	*APP*	-10.88
B10	*BECN1*	-10.76

Array position	Gene	Amount down-regulated
F06	*PIK3R4*	-10.62
G09	*ULK1*	-10.39
C07	*CTSD*	-10.31
E02	*HSPA8*	-10.22
A07	*ATG16L2*	-10.19
B05	*BAD*	-10.15
C02	*CASP8*	-10.11
F05	*PIK3CG*	-10.06

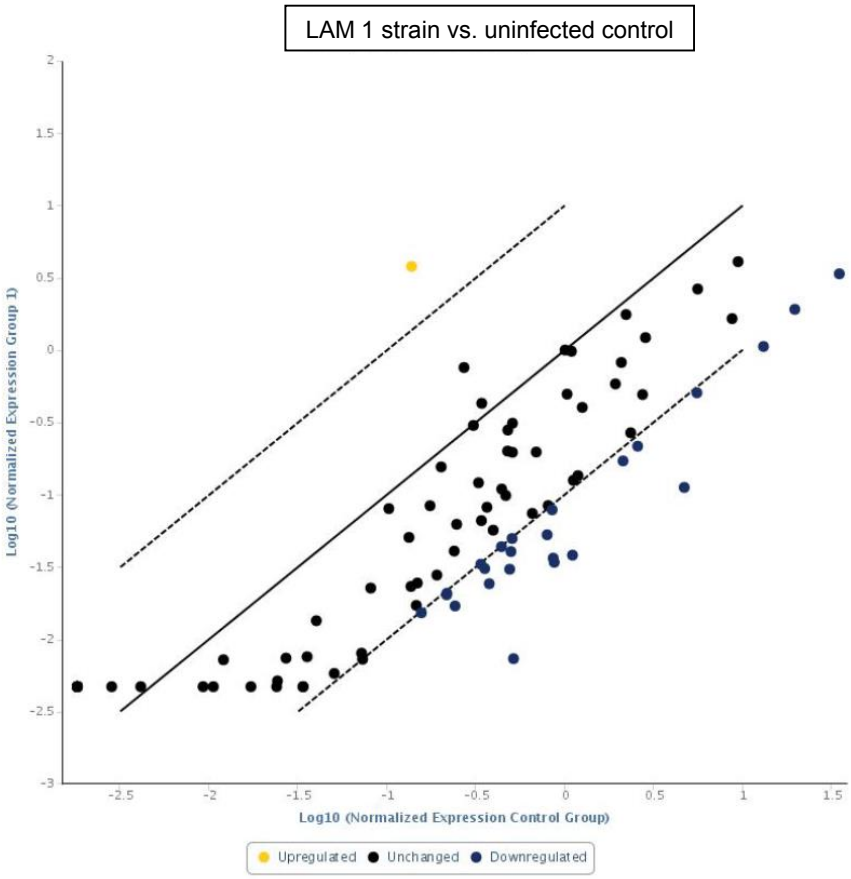

Figure 3.2. Scatterplot of control group vs. LAM 1 with a 10-fold regulation threshold.

Infection of THP-1 cells with the LAM 1 strain resulted in the differential expression of several autophagy-related genes. *TNF, CDKN1B, RGS19, FADD, HTT, ATK1, TGFB1,*

BCL2L1, HDAC1, APP, BECN1, ULK1, BAD, CASP8 and *PIK3CG* are co-regulators of autophagy and apoptosis, while *CDKN1B* and *TGFB1* are co-regulators of the cell cycle. *LAMP1* induces autophagy by intracellular pathogens and links the autophagosome to the lysosome and *GAA, MAPK14, HGS, PIK3R4, CTSD* induces autophagy in response to intracellular signals. *ATG9A* and *ATG4C* are involved in autophagic vacuole formation and *ATG9A, ATG4C* and *ATG16L2* in protein transport. *ATG4C* further influences protease activity and protein targeting to membrane/vacuole and *HSPA8* influences chaperone-mediated autophagy.

3.2.2 Typical Beijing strain

Table 3.3. Down-regulated genes, in THP-1 cell infected with a Typical Beijing (sub-lineage 4) *M.tb* strain compared to uninfected cells.

DOWN- REGULATED (10-fold)		
Array position	**Gene**	**Amount down-regulated**
F12	*RPS6KB1*	-22.50

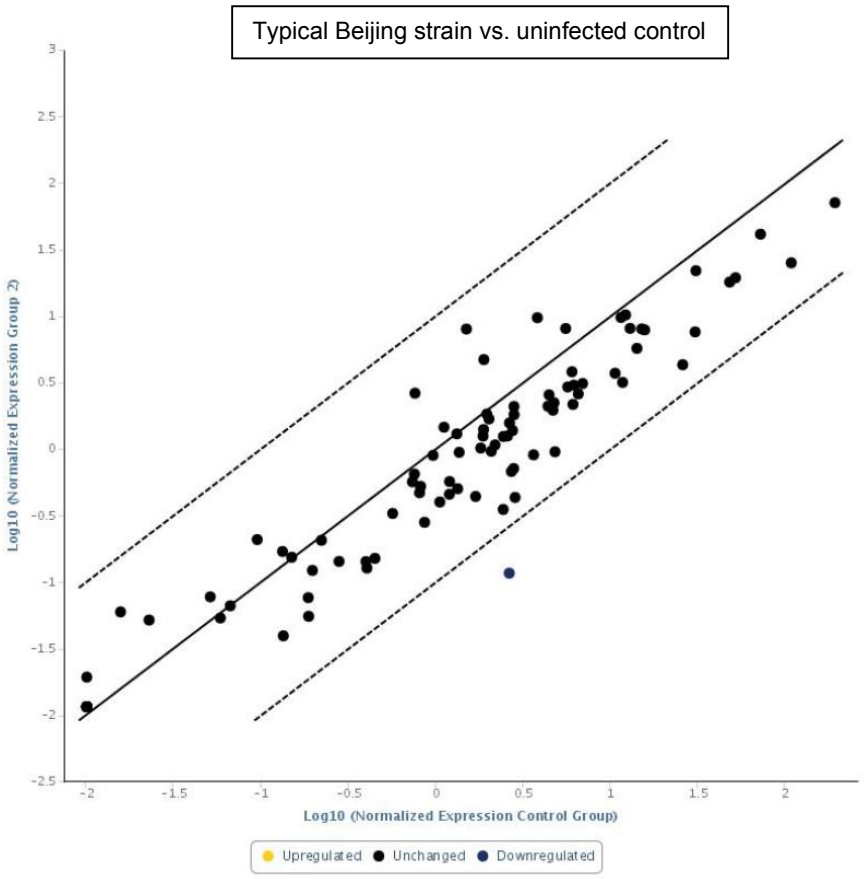

Figure 3.3. Scatterplot of control group vs. Typical Beijing with a 10-fold regulation threshold.

The only autophagy-related gene that was differentially expressed more than 10-fold following the infection of THP-1 cells with the typical Beijing strain was *RPS6KB1*. The protein encoded by this gene is regulated by the mTOR complex and associated with the initiation of mRNA translation and the control of protein synthesis.

3.2.3 Atypical Beijing strain

Table 3.4. Down-regulated genes, in THP-1 cell infected with an Atypical Beijing (sub-lineage 1) *M.tb* strain compared to uninfected cells.

DOWN- REGULATED (10-fold)		
Array position	**Gene**	**Amount down-regulated**
C03	*CDKN1B*	-18.61

Array position	Gene	Amount down-regulated
D04	*FADD*	-16.00
A03	*APP*	-11.75
D06	*GAA*	-11.55

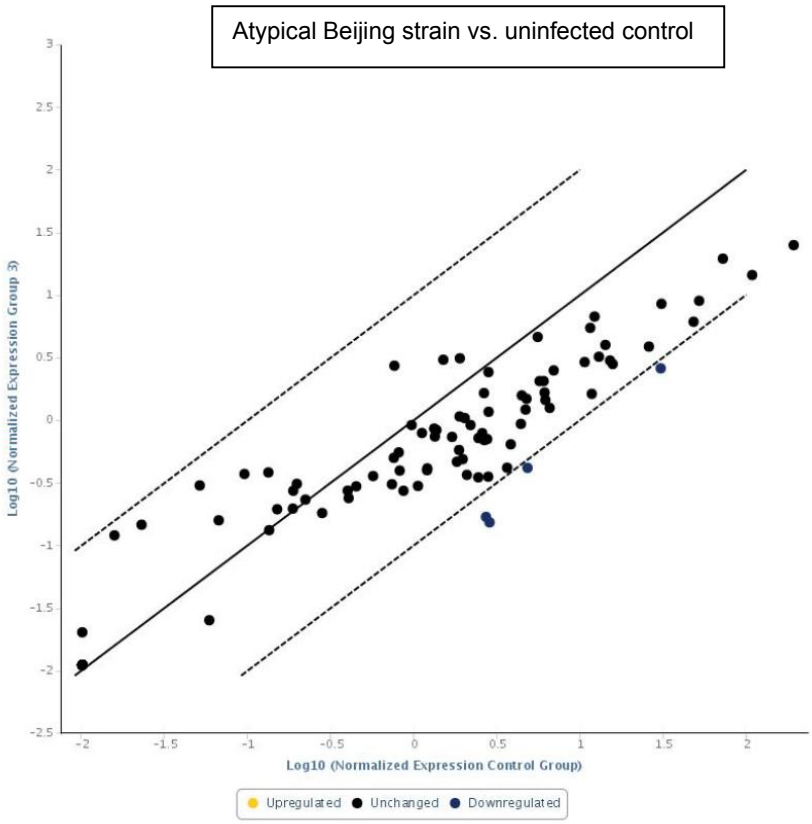

Figure 3.4. Scatterplot of control group vs. Atypical Beijing with a 10-fold regulation threshold.

None of the investigated genes were found to be up-regulated more than 10-fold in cells infected with the typical Beijing strain. Four genes were found to be down-regulated. These include *CDKN1B, FADD* and *APP*, co-regulators of autophagy and apoptosis and *GAA* which is plays a role in autophagy induction in response to other intracellular signals.

3.2.4 Laboratory strain H37Rv

Table 3.5. Up- and down-regulated genes, in THP-1 cell infected with an H37Rv laboratory *M.tb* strain compared to uninfected cells.

UP- REGULATED (10-fold)		
Array position	**Gene**	**Amount up-regulated**
G06	*TNF*	+30.87
DOWN- REGULATED (10-fold)		
Array position	**Gene**	**Amount down-regulated**
G08	*TP53*	-11.63
D06	*GAA*	-10.01

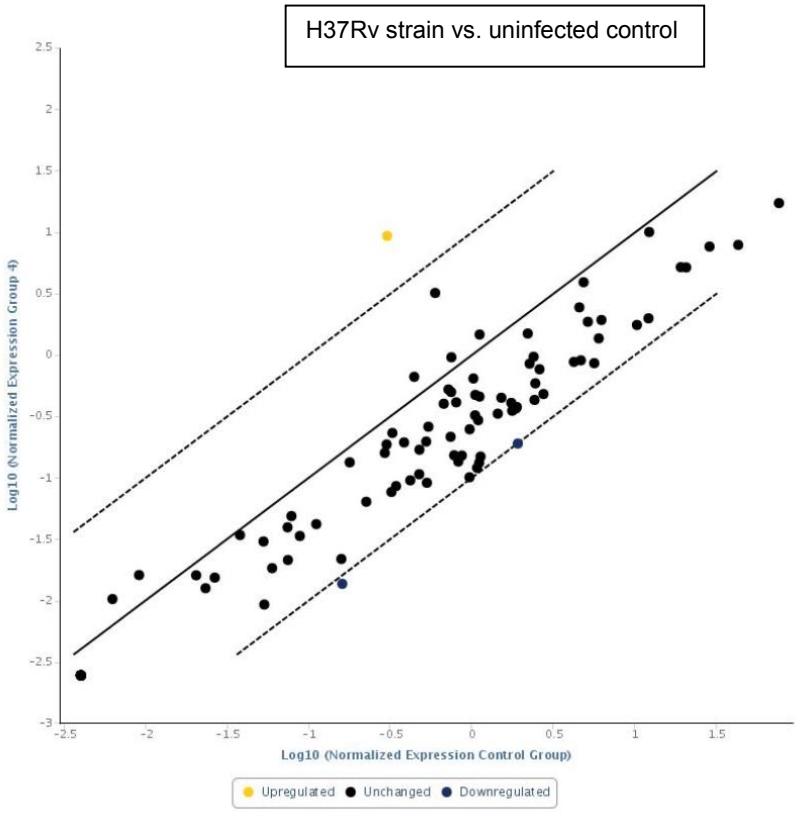

Figure 3.5. Scatterplot of control group vs. H37Rv with a 10-fold regulation threshold.

Three genes were differentially expressed in THP-1 cells infected with H37Rv. *TNF* and *TP53* are co-regulators of autophagy and apoptosis. *GAA* can induce autophagy in response to other intracellular signals.

3.2.5 CAS/Kili strain

Table 3.6. Up-regulated genes, in THP-1 cell infected with a CAS/Kili *M.tb* strain compared to uninfected cells.

UP- REGULATED (10-fold)		
Array position	**Gene**	**Amount up-regulated**
G06	*TNF*	+176.95
G04	*TGM2*	+13.93

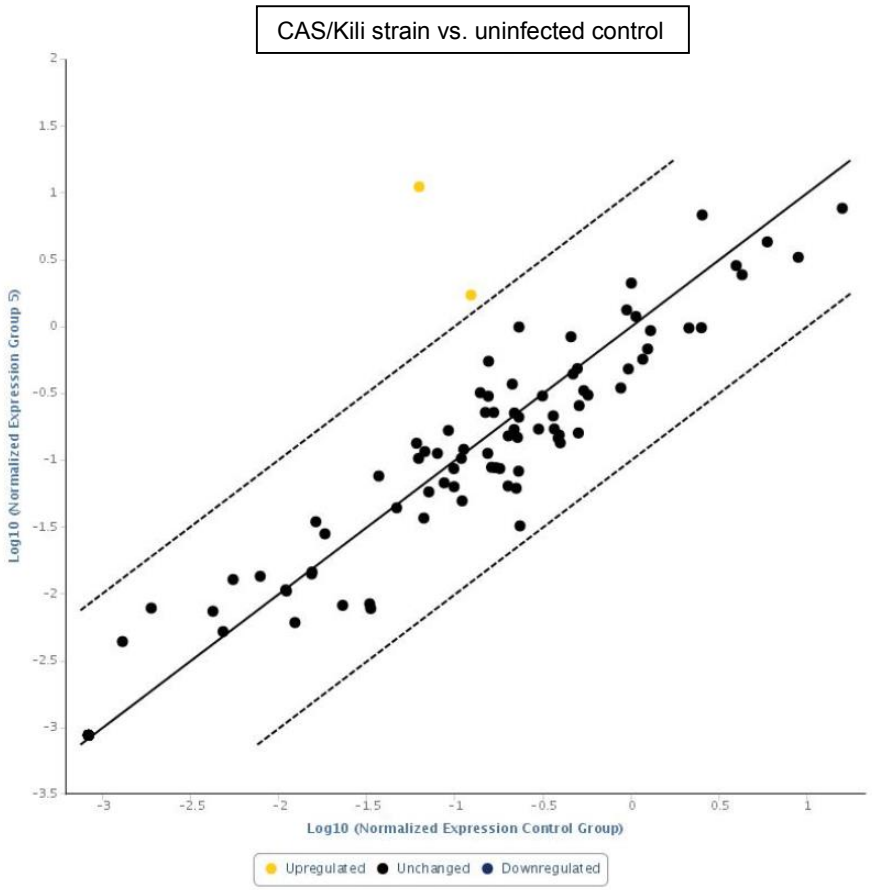

Figure 3.6. Scatterplot of control group vs. CAS/Kili with a 10-fold regulation threshold.

None of the investigated genes were down-regulated more that 10-fold when THP-1 cells when infected with the CAS/Kili strain. *TNF* and *TGM2* were up-regulated more than 10-fold.

3.2.6 LCC strain

Table 3.7. Up- and down-regulated genes, in THP-1 cell infected with a LCC (4 bander) *M.tb* strain compared to uninfected cells.

UP- REGULATED (10-fold)		
Array position	**Gene**	**Amount up-regulated**
E04	*IFNG*	+81.11
C04	*CDKN2A*	+35.43
G10	*ULK2*	+17.04
E07	*IRGM*	+11.70
DOWN- REGULATED (10-fold)		
Array position	**Gene**	**Amount down-regulated**
G02	*SQSTM1*	-5 656.33
C07	*CTSD*	-1 004.24
G03	*TGFB1*	-606.65
G12	*WIPI1*	-345.83
F11	*RGS19*	-215.41
C12	*DRAM2*	-170.53
E11	*MAPK14*	-143.75
C08	*CTSS*	-108.18
D12	*HGS*	-61.17
C06	*CTSB*	-56.51
F12	*RPS6KB1*	-42.51
E10	*MAP1LC3B*	-41.56
C05	*CLN3*	-40.60
Array position	**Gene**	**Amount down-regulated**
E08	*LAMP1*	-37.53
D10	*HDAC1*	-36.47
F10	*RB1*	-31.55
E01	*HSP90AA1*	-27.45
D11	*HDAC6*	-24.23

F07	*PRKAA1*	-22.88
E12	*MAPK8*	-18.28
D02	*EIF4G1*	-17.95
D09	*GABARAPL2*	-17.77
C02	*CASP8*	-17.16

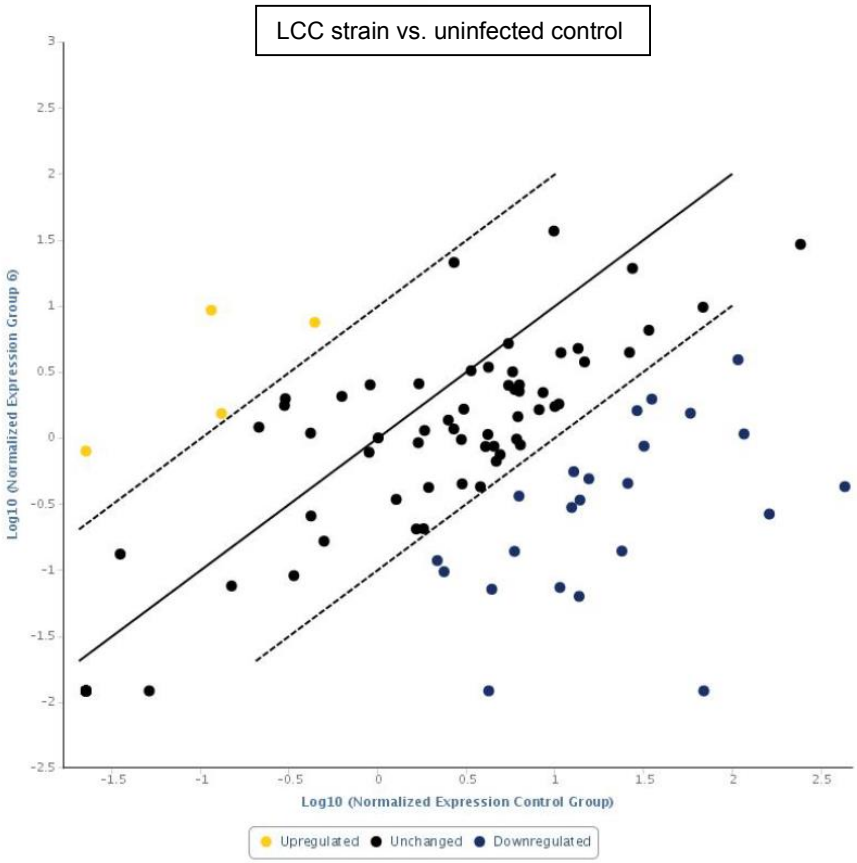

Figure 3.7. Scatterplot of control group vs. LCC with a 10-fold regulation threshold.

Infection of THP-1 with the LCC strain resulted in the up-regulation of *INFG, CDKN2A, ULK2* and *IRGM.* Several genes were down-regulated (table 3.7) following infection.

TGFB1, IFNG, SQSTM1, WIPI1, CLN3, CASP8, HDAC1, MAPK8, PRKAA1 and *CDKN2A* are co-regulators of autophagy and apoptosis and *RB1* regulates the cell cycle; *IFNG* and *LAMP1* can also induce autophagy by intracellular pathogens; and *MAP1LC3B, GABARAPL2, RGS19* and *IRGM* are involved in the formation of the autophagic vacuole.

ULK2, MAPK14, CTSS, CTSD, RPS6KB1, LAMP1, EIF4G1 and *DRAM2* induces autophagy in response to other intracellular signals. *HSP90AA1* influences the chaperone-mediated autophagy. *HDAC6* is one of the genes involved in protein ubiquitination, while *GABARAPL2* is responsible for protein transport.

3.2.7 Haarlem 3 strain

Table 3.8. Up- and down-regulated genes, in THP-1 cell infected with a Haarlem 3 (F4) *M.tb* strain compared to uninfected cells.

UP- REGULATED (10-fold)		
Array position	**Gene**	**Amount up-regulated**
C04	*CDKN2A*	+98.21
E04	*IFNG*	+90.88
F09	*RAB24*	+66.13
E07	*IRGM*	+38.15
G05	*TMEM74*	+32.42
E06	*INS*	+20.43
D03	*ESR1*	+13.84
G10	*ULK2*	+12.36
DOWN- REGULATED (10-fold)		
Array position	**Gene**	**Amount down-regulated**
G02	*SQSTM1*	-3 021.77
C07	*CTSD*	-918.43
G03	*TGFB1*	-361.96
Array position	**Gene**	**Amount down-regulated**
F12	*RPS6KB1*	-258.28
C12	*DRAM2*	-126.81
E11	*MAPK14*	-93.30
C08	*CTSS*	-59.53
F11	*RGS19*	-55.99
C06	*CTSB*	-55.96

B11	*BID*	-38.66
E08	*LAMP1*	-30.18
D10	*HDAC1*	-29.46
B12	*BNIP3*	-28.72
D09	*GABARAPL2*	-23.98
C05	*CLN3*	-23.08
E10	*MAP1LC3B*	-21.55
C11	*DRAM1*	-19.32
D12	*HGS*	-15.43
E01	*HSP90AA1*	-14.05
D11	*HDAC6*	-11.98
F07	*PRKAA1*	-11.86
E12	*MAPK8*	-11.83

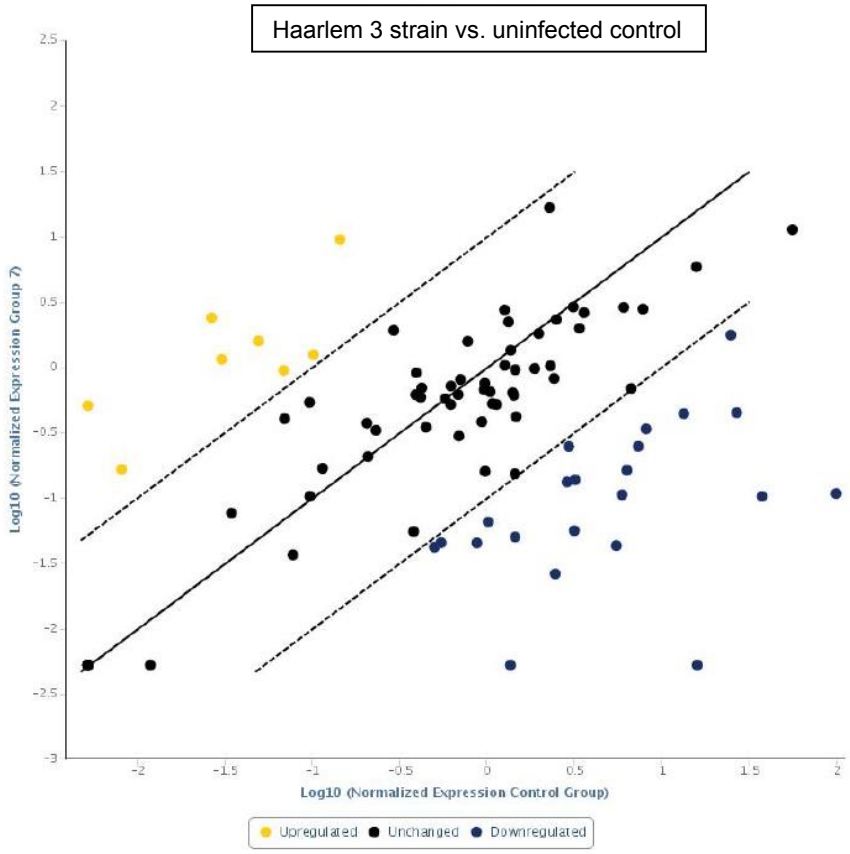

Figure 3.8. Scatterplot of control group vs. Haarlem 3 with a 10-fold regulation threshold.

A number of genes were differentially expressed more than 10-fold in THP-1 cells infected with the Haarlem 3 strain. The genes *TGFB1, IFNG, SQSTM1, WIPI1, CLN3, CTSB, CASP8, HDAC1, MAPK8, PRKAA1, BNIP3, DRAM1* and *CDKN2A* are co-regulators of autophagy and apoptosis and *RB1* regulates the cell cycle; *IFNG* and *LAMP1* can also induce autophagy by intracellular pathogens; and *MAP1LC3B, GABARAPL2, RGS19* and *IRGM* are involved in the formation of the autophagic vacuole. *ULK2, MAPK14, CTSS, CTSD, RPS6KB1, LAMP1, EIF4G1* and *DRAM2* induces autophagy in response to other intracellular signals. *HSP90AA1* influences the chaperone-mediated autophagy. *HDAC6* is one of the genes involved in protein ubiquitination, while *GABARAPL2* is responsible for protein transport. *DRAM1* links autophagosomes to lysosomes (“RT2 Profiler PCR Arrays - QIAGEN Online Shop,”).

3.2.8 Typical Beijing strain vs. Atypical Beijing strain

When comparing differential gene expression in cells infected with the Typical and Atypical Beijing strains, two closely related strains, a number of genes were differentially expressed (figure 3.9, Table 3.9).

Table 3.9. Differential gene expression of THP-1 cells infected with a Typical Beijing (sub-lineage 4) *M.tb* strain compared to THP-1 cell infected with Atypical Beijing (sub-lineage 1) *M.tb* strain.

Layout	1	2	3	4	5	6	7	8	9	10	11	12
A	AKT1 -1.82	AMBRA1 -1.21	APP -2.73	ATG10 2.55 B	ATG12 -2.11	ATG16L1 -1.10	ATG16L2 1.04	ATG3 -1.73	ATG4A 1.20	ATG4B -1.24	ATG4C 1.57	ATG4D -1.42
B	ATG5 -14.13	ATG7 -1.49	ATG9A -2.46	ATG9B 1.36	BAD -2.02	BAK1 -1.51	BAX -1.94	BCL2 -2.02	BCL2L1 -1.82	BECN1 -1.51	BID -1.42	BNIP3 1.24
C	CASP3 -2.04	CASP8 -1.89	CDKN1B -2.65	CDKN2A 1.12	CLN3 -1.94	CTSB -1.67	CTSD -2.66	CTSS -2.02	CXCR4 -1.72	DAPK1 3.59	DRAM1 1.79	DRAM2 -1.19
D	EIF2AK3 2.10	EIF4G1 -2.35	ESR1 2.41	FADD -3.75	FAS 2.76	GAA -2.14	GABARAP -2.49	GABARAPL1 -1.06	GABARAPL2 -2.62	HDAC1 -1.33	HDAC6 -1.25	HGS -3.50
E	HSP90AA1 -2.75	HSPA8 -1.63	HTT -2.10	IFNG 4.16	IGF1 5.27	INS 2.15	IRGM -1.98	LAMP1 -1.04	MAP1LC3A -1.51	MAP1LC3B -1.63	MAPK14 -1.41	MAPK8 1.09
F	MTOR -1.72	NFKB1 -1.45	NPC1 -1.22	PIK3C3 -1.61	PIK3CG 1.06	PIK3R4 -1.07	PRKAA1 -1.33	PTEN 1.17	RAB24 1.36	RB1 -1.16	RGS19 -1.22	RPS6KB1 15.10
G	SNCA 3.02	SQSTM1 -2.40	TGFB1 -1.98	TGM2 -2.45	TMEM74 1.91	TNF 1.11	TNFSF10 2.05	TP53 2.01	ULK1 -1.29	ULK2 2.71	UVRAG 1.26	WIPI1 -1.41

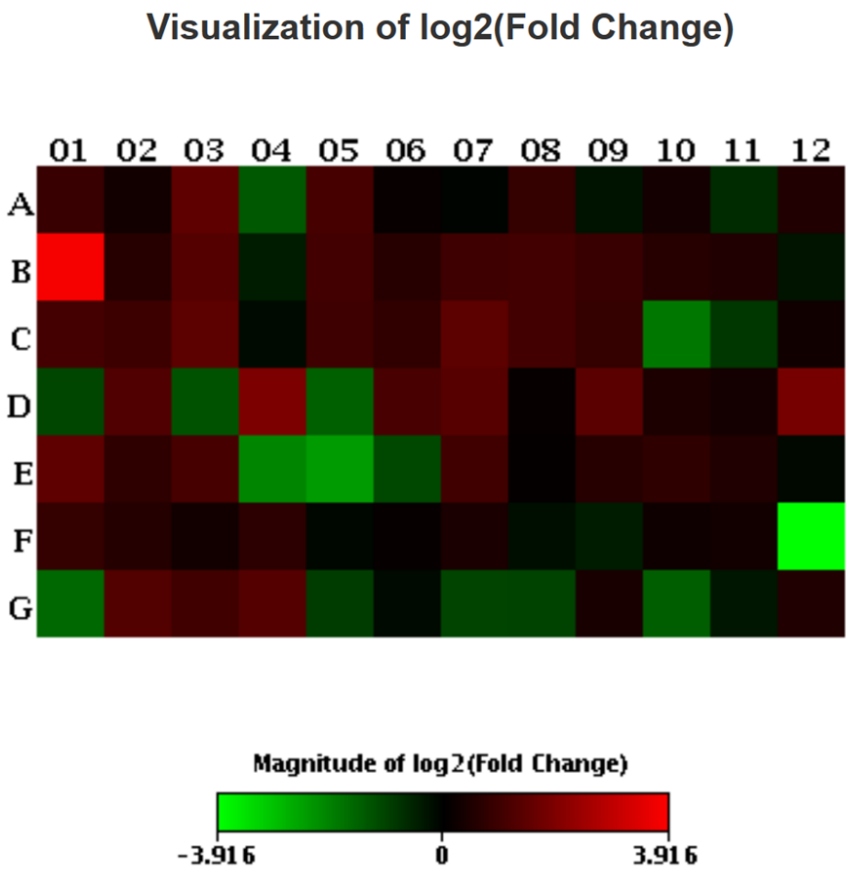

Figure 3.9. Heat map of differential gene expression of typical Beijing vs. atypical Beijing.

3.2.9 Typical Beijing strain vs. CAS/Kili strain

We then compared autophagy associated gene expression differences in THP-1 cells infected the highly virulent Typical Beijing strain versus the moderately virulent CAS/Kili strain. Compared to the CAS/Kili strain, infection of THP-1 cells with the typical Beijing strain resulted in the down-regulation of several genes (Table 3.10).

Table 3.10. Differential gene expression of THP-1 cells infected with a Typical Beijing (sub-lineage 4) *M.tb* strain compared to THP-1 infected with a CAS/Kili strain.

Layout	1	2	3	4	5	6	7	8	9	10	11	12
A	AKT1 -1.58	AMBRA1 -1.65	APP -1.34	ATG10 -2.00	ATG12 -1.49	ATG16L1 1.19	ATG16L2 -2.13	ATG3 -1.32	ATG4A -1.42	ATG4B -2.29	ATG4C -1.02	ATG4D 1.22
B	ATG5 3.09	ATG7 -3.07	ATG9A 1.06	ATG9B 2.42	BAD 1.07	BAK1 1.43	BAX -1.33	BCL2 -1.96	BCL2L1 -1.11	BECN1 1.29	BID -2.15	BNIP3 -1.04
C	CASP3 -2.32	CASP8 -1.20	CDKN1B 1.29	CDKN2A 2.13	CLN3 1.14	CTSB -1.42	CTSD -1.12	CTSS -1.31	CXCR4 -1.19	DAPK1 -2.76	DRAM1 -7.55	DRAM2 1.03
D	EIF2AK3 -5.24	EIF4G1 -1.53	ESR1 1.53	FADD 1.06	FAS -1.92	GAA -1.47	GABARAP 1.13	GABARAPL1 -1.33	GABARAPL2 -1.23	HDAC1 -1.04	HDAC6 -1.76	HGS 1.99
E	HSP90AA1 -1.65	HSPA8 -1.36	HTT -1.06	IFNG 1.01	IGF1 -2.73	INS 1.31	IRGM -1.01	LAMP1 -2.34	MAP1LC3A -1.40	MAP1LC3B -1.08	MAPK14 1.39	MAPK8 -1.30
F	MTOR -2.45	NFKB1 -5.67	NPC1 -2.23	PIK3C3 -1.27	PIK3CG -1.89	PIK3R4 -1.96	PRKAA1 -1.56	PTEN -1.40	RAB24 1.68	RB1 -1.03	RGS19 1.31	RPS6KB1 -15.10
G	SNCA -1.57	SQSTM1 -3.25	TGFB1 -1.08	TGM2 -2.24	TMEM74 1.49	TNF -43.83	TNFSF10 1.63	TP53 1.58	ULK1 -1.16	ULK2 -2.94	UVRAG 1.23	WIPI1 -1.21

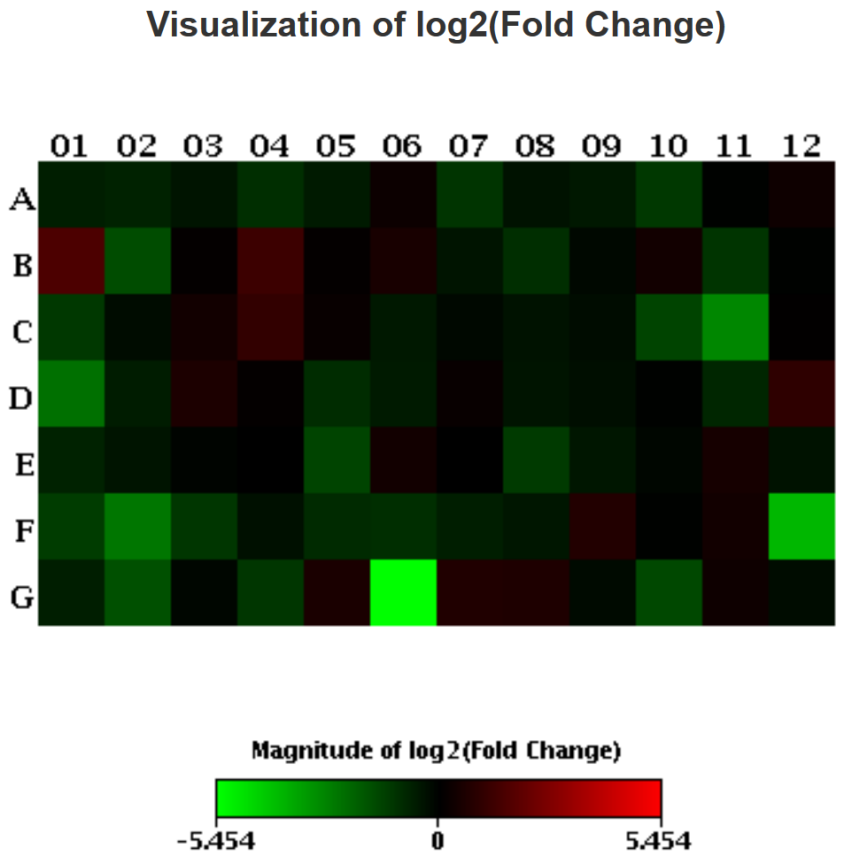

Figure 3.10. Heat map of differential gene expression of Typical Beijing vs. CAS/Kili.

3.2.10. LCC versus Haarlem 3

Interestingly, when comparing differential gene expression in cells infected with the LCC strain to that of cells infected with the Haarlem 3 strain, several of the same genes were found to be differentially expressed in both. These genes can be seen in table 3.11. This may indicate that these two strains are able to evade the autophagy pathway using a common mechanism.

Table 3.11. This table indicates which genes are similarly regulated, with a fold change value of 10 or more, in the LCC and Haarlem 3 strains.

Gene	*M.tb* strain		Function of the gene(s)
	Fold change values of the strain of interest compared to the uninfected control		
	LCC	Haarlem 3	
CDKN2A	+35.43	+98.21	Co-Regulator of Autophagy and Apoptosis; Co-Regulator of Autophagy and the Cell Cycle
IFNG	+81.11	+90.88	Co-Regulator of Autophagy and Apoptosis; Co-Regulator of Autophagy and the Cell Cycle; Autophagy Induction by Intracellular pathogens
ULK2	+17.04	+12..36	Autophagy in Response to Other Intracellular Signals
IRGM	+11.70	+38.15	Genes Involved in Autophagic Vacuole Formation
LAMP1	-37.53	-30.18	Gene Responsible for Protein Transport; Autophagy Induction by Intracellular Pathogens
RGS19	-215.41	-55.99	Genes Involved in Autophagic Vacuole Formation
MAPK14	-143.75	-93.3	Autophagy in Response to Other Intracellular Signals
SQSTM1	-5 656.33	-3 021.77	Co-Regulator of Autophagy and Apoptosis
CTSD	-1 004.24	-918.43	Autophagy in Response to Other Intracellular Signals

Gene	*M.tb* strain		Function of the gene(s)
	Fold change values of the strain of interest compared to the uninfected control		
	LCC	Haarlem 3	
TGFB1	-606.65	-361.96	Co-Regulator of Autophagy and Apoptosis; Co-Regulator of Autophagy and the Cell Cycle
DRAM2	-170.53	-126.81	Autophagy in Response to Other Intracellular Signals
CTSS	-108.18	-59.53	Autophagy in Response to Other Intracellular Signals
CTSB	-56.51	-55.96	Co-Regulator of Autophagy and Apoptosis
RPS6KB1	-42.51	-258.28	Autophagy in Response to Other Intracellular Signals
MAP1LC3B	-41.56	-21.55	Genes Involved in Autophagic Vacuole Formation
CLN3	-40.6	-23.08	Co-Regulator of Autophagy and Apoptosis
HDAC1	-36.47	-29.46	Co-Regulator of Autophagy and Apoptosis
MAPK8	-18.28	-11.83	Co-Regulator of Autophagy and Apoptosis
PRKAA1	-22.88	-11.86	Co-Regulator of Autophagy and Apoptosis
HDAC6	-24.23	-11.86	Genes involved in Protein Ubiquitination
HSP90AA1	-27.45	-14.05	Chaperone-mediated Autophagy
HGS	-61.17	-15.43	Autophagy in Response to Other Intracellular Signals
GABARAPL2	-17.77	-23.98	Genes Involved in Autophagic Vacuole Formation; Genes Responsible for Protein Targeting to Membrane/Vacuole

3.2.11. Induction of autophagy by different *M.tb* strains

In order to investigate the levels of autophagy induction by each of the investigated strains, the expression of *IFNG*, which is known to play a crucial role in autophagy induction (Gutierrez et al., 2004) was compared between cells infected with each of the

strains. The results of this comparison is shown in figure 3.11. Interestingly, the typical and atypical Beijing strains, the LCC strain and the Haarlem 3 strain significantly regulated *IFNG* expression, while the less virulent CAS/Kili had down-regulated *IFNG* expression.

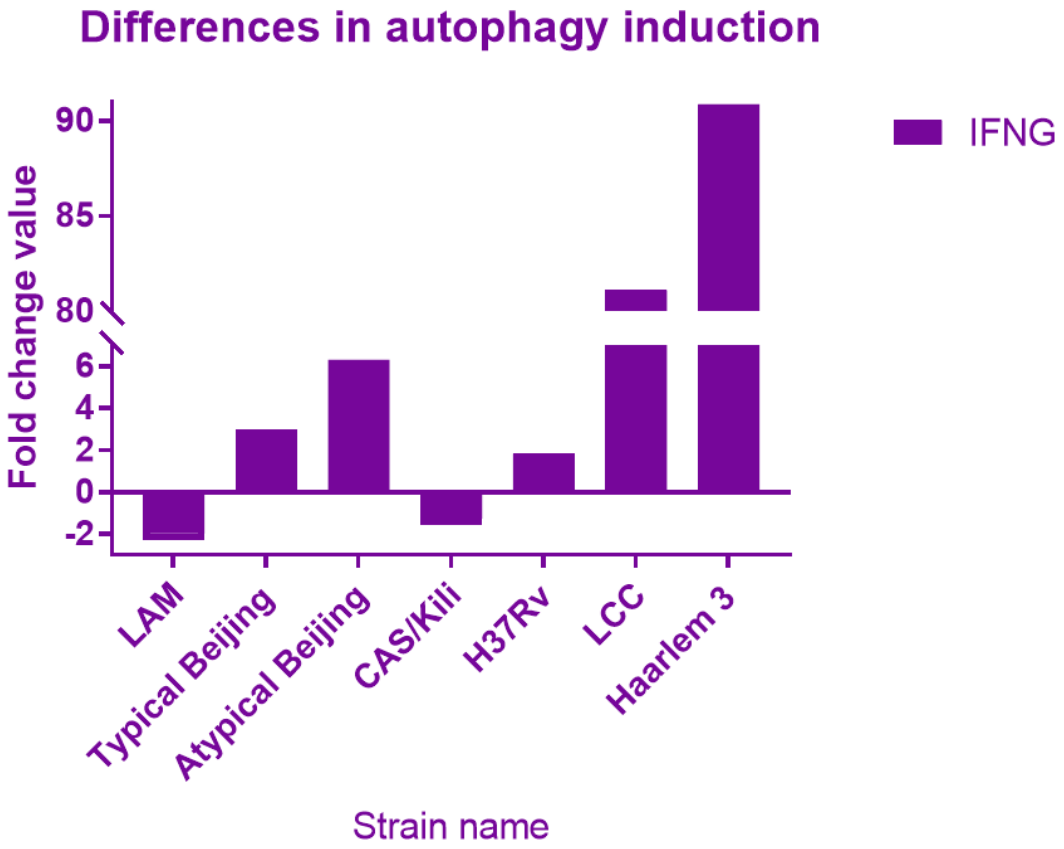

Figure 3.11. Induction of autophagy differences between the *M.tb* strains, measured by the fold change of *IFNG*.

3.2.12. Differences in formation of autophagosomes

The genes that play a role in the formation of the autophagosome were investigated to determine if any of the strains can inhibit or induce the formation of this vesicle. The genes that play a role in the formation are *ATG1-10, ATG12-14* and *ATG16-18* (Kawamata et al., 2008). The results can be observed in figure 3.12. The LAM 1, Atypical Beijing, H37Rv, CAS/Kili and LCC strains have the ability to inhibit autophagosome formation, whereas Typical Beijing and Haarlem 3 induces the formation of autophagosomes.

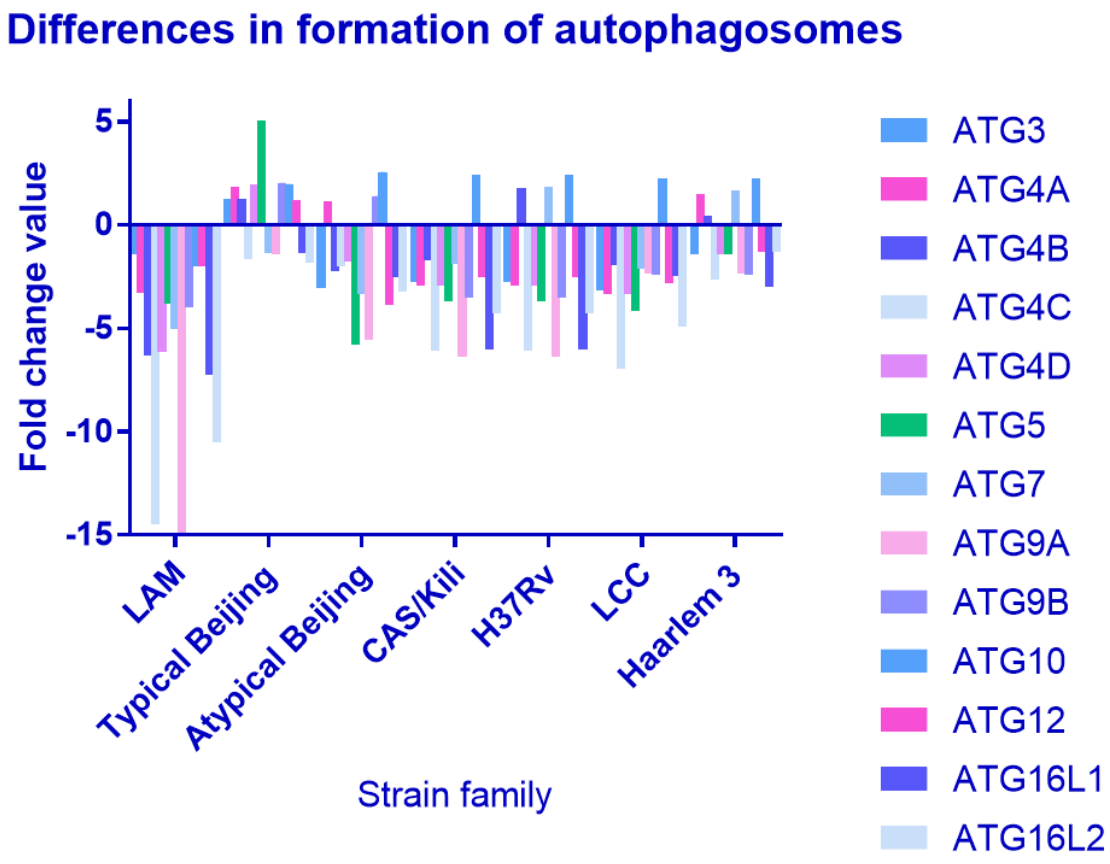

Figure 3.12. The differences between the *M.tb* strains in the formation of autophagosomes, measured by the fold change of the genes involved in the process.

3.2.13. Differences in formation of autophagolysosomes

To determine whether there were any differences in the ability of each strain to inhibit autophagolysosomal fusion, the expression genes involved in autophagolysomal fusion, *LAMP1*, *DRAM*, *GABARAP* and *NPC1* was compared between cells infected with the various *M.tb* strains (figure 3.13). None of the strains caused the up-regulation of either *LAMP1* or *GABARAP.* In addition to this, each strain down-regulated at least two of these genes which may indicate that each strain is able to block autophagosomal fusion. This result is not unexpected since it is known that *M.tb* is able to block autophagolysomal fusion.

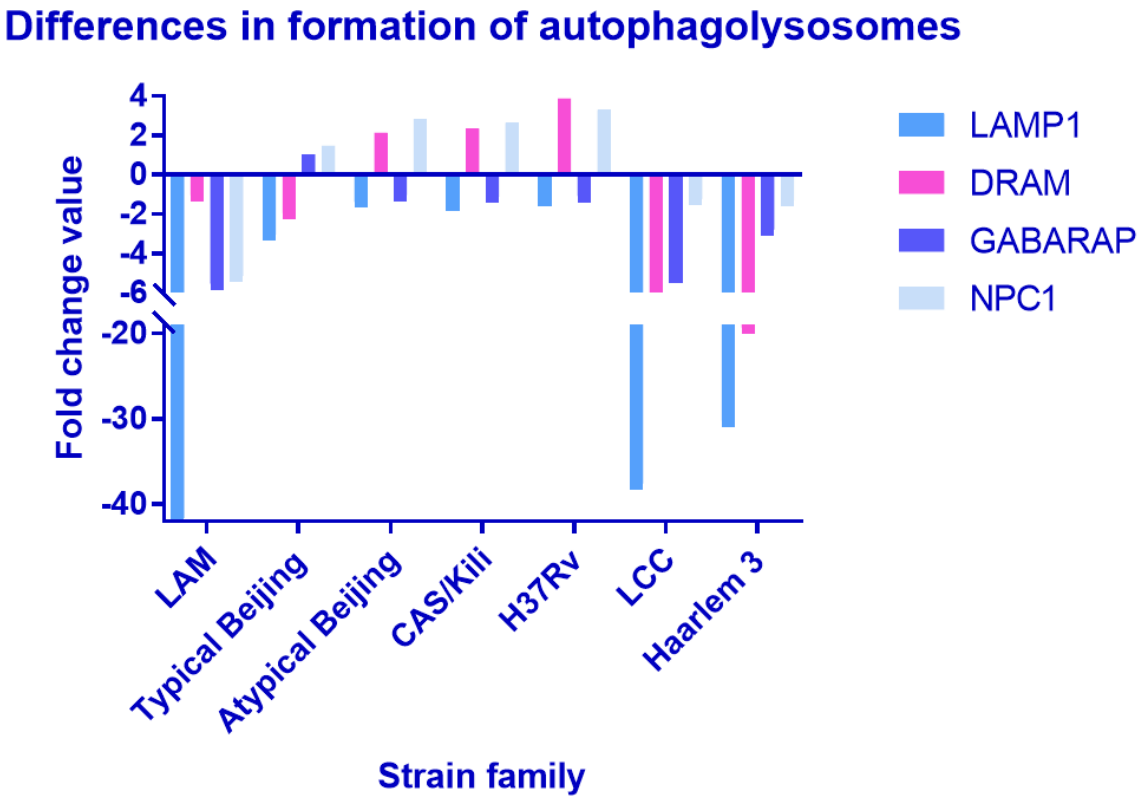

Figure 3.13. The differences between the *M.tb* strains in the formation of autophagolysosomes, measured by the fold change of the genes *LAMP1, DRAM*, *GABARAP* and *NPC1*.

In summary, infection of THP-1 cells with different *M.tb* strains resulted in the differential expression of a number of autophagy associated genes. Many of the genes that were differentially expressed in the cells infected with were genes involved in autophagolysosomal formation, in the formation of the autophagic vacuole, protein transport and chaperone-mediated autophagy. Genes differentially expressed in response to infection with Typical and Atypical Beijing strains play important roles in autophagy induction in response to other intracellular signals, while the laboratory strain, H37Rv and the CAS/Kili induce differential expression of genes involved with the co-regulation of autophagy. The LCC strain can successfully control the autophagic vacuole formation. In addition to this, the Haarlem 3 strain can influence the fusion of the

autophagosome with the lysosome. All seven strains are successful in controlling and regulating autophagy, apoptosis and the cell cycle.

3.3 CASE-CONTROL ASSOCIATION STUDY

In the present study, we hypothesized that genes associated with autophagy that are significantly up- or down-regulated following infection with mycobacteria can be considered plausible candidate genes for TB susceptibility. For this reason, polymorphisms in the up- and down-regulated genes described in section 3.2 were evaluated for association with TB susceptibility in our TB case-control cohort (section 2.5). The polymorphisms investigated were genotyped as part of a larger study using the Illumina MEGA® genotyping array.

Before correction for multiple testing, a number of SNPs were nominally associated with TB susceptibility. However, following Bonferonni correction for multiple testing, no association was observed for any of the investigated SNPs (table 3.12).

Table 3.12. The list of SNPs that had statistically significant unadjusted p-values and their respective Bonferonni corrected p-values, together with their Odds ratio and 95% Confidence interval.

CHR	SNP	rs number	UNADJ	BONF	OR	L95	U95
11	11:618051	rs749469151	0.003191	1	4.608	1.669	12.72
11	11:1325388	rs5743891	0.004159	1	0.4577	0.2682	0.7811
11	11:1355581	rs61869688	0.005597	1	0.4526	0.2583	0.793
11	11:1988393	rs61866962	0.005772	1	0.5839	0.3985	0.8555
11	11:1372980	rs61869716	0.006542	1	0.4743	0.277	0.812
11	11:1912726	rs542605	0.01314	1	1.338	1.063	1.683
11	11:1677351	rs10400297	0.01337	1	0.6367	0.4453	0.9105
19	19:41851509	rs4803455	0.01426	1	0.7529	0.5999	0.9447
11	11:365636	rs11246130	0.01714	1	0.7531	0.5965	0.9509
11	11:2006183	rs55941855	0.01741	1	2.81	1.199	6.583

CHR	SNP	rs number	UNADJ	BONF	OR	L95	U95
11	11:1945153	rs148051896	0.01919	1	0.3985	0.1845	0.8607
17	17:79663137	rs115492948	0.0213	1	3.126	1.185	8.248
11	11:882406	rs61869681	0.02204	1	0.6146	0.4051	0.9323
9	9:21984661	rs3731217	0.02253	1	1.547	1.063	2.252
5	5:40770220	rs1645491	0.02729	1	1.288	1.029	1.612
11	11:1258803	rs2014486	0.02874	1	1.28	1.026	1.596
11	11:788007	rs6597982	0.0294	1	0.7598	0.5934	0.9729
11	11:1667746	rs16927442	0.02953	1	1.414	1.035	1.932
13	13:113975290	rs9549739	0.03152	1	1.375	1.029	1.837
11	11:1272754	rs3021156	0.03156	1	0.4459	0.2135	0.9311
1	1:150724296	rs113695437	0.03185	1	2.148	1.069	4.318
11	11:1263776	rs2943510	0.03185	1	0.7213	0.5352	0.972
11	11:1767225	rs551749595	0.03298	1	2.585	1.08	6.19
5	5:40759997	rs114336915	0.03341	1	0.4731	0.2374	0.9429
11	11:351629	rs76020902	0.03443	1	1.417	1.026	1.957
11	11:1940543	rs57253033	0.03541	1	0.7714	0.6056	0.9824
11	11:1142621	rs56199221	0.03541	1	0.6852	0.4818	0.9745
11	11:2019174	rs76989783	0.03626	1	0.6267	0.4047	0.9705
11	11:1075920	rs11825977	0.03782	1	1.379	1.018	1.866
11	11:2141603	rs4341514	0.03788	1	0.7821	0.6202	0.9863
11	11:2078654	rs114401323	0.03825	1	2.735	1.056	7.086
11	11:825110	rs1138714	0.03855	1	1.314	1.014	1.702
11	11:1941465	rs113420515	0.03946	1	2.921	1.053	8.102
12	12:12870695	rs34330	0.03949	1	0.7871	0.6268	0.9885
6	6:36073363	rs60458591	0.044	1	0.5982	0.3629	0.9863
22	22:18247811	rs112079353	0.04637	1	1.405	1.005	1.964
11	11:1093945	rs7934606	0.04845	1	1.342	1.002	1.796
5	5:40771216	rs257009	0.0499	1	1.258	1	1.584

CHR = Chromosome, UNADJ = Unadjusted, asymptotic significance value, BONF = Bonferroni adjusted significance value, OR = Odds ratio, L95 = Lower 95% interval, U95 = Upper 95% interval.

Chapter Four

Discussion

CHAPTER FOUR
DISCUSSION

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Discussion

M.tb, the causative agent of tuberculosis infects one third of the world's population causing approximately 1.8 million deaths per year (World Health Organisation, 2015). One of the major determinants of *M.tb* pathogenicity is the ability of the bacterium to survive within host macrophages (Hingley-Wilson *et al*., 2003). Although many factors have been shown to be involved in *M.tb's* survival within the host, the mammalian autophagy pathway is now recognised as a major factor determining the course of infection (Zullo and Lee, 2011).

Autophagy is a catabolic process that degrades undesirable cytosolic components (He and Klionksy, 2009). In addition to this, autophagy has also been found to play a significant role in the destruction of intracellular pathogens like *M.tb* (Deretic, 2011). The autophagic elimination of mycobacteria occurs when a LC3-IIB positive autophagosome containing the bacteria fuse with a lysosome and, in so doing, exposing the pathogen to proteases and anti-microbial peptides (Guiterrez *et al*., 2004, Ponpuak *et al*., 2011).

Interestingly, a number of independent human genetic studies have found an association between the *IGRM* gene, which encodes an autophagy-modulating factor and increased susceptibility to TB (Intemann *et al*., 2009; Che *et al*., 2010; King *et al*., 2011; Bahari *et al*., 2012). Moreover, mice that lack the expression of the *IRGM* orthologue, LRG47, are unable to control *M.tb* and rapidly succumb to infection (MacMicking *et al*., 2003). In addition, polymorphisms in the gene encoding the vitamin D receptor has been associated with susceptibility to TB when combined with low concentrations of calcitriol (1,25 dihydroxyvitamin D3) which is a precursor to active form of vitamin D (Wilkonson., *et* al., 2000). Calcitriol has recently been functionally linked to the autophagic elimination of *M.tb* (Yuk *et al*., 2009; Shin *et al*., 2010a; Campbell and Spector, 2012).

The importance of autophagy in host cell response to *M.tb* infection is further highlighted by the fact that virulent mycobacteria have evolved specific mechanisms to inhibit

autophagy (Kumar *et al*., 2010). Studies have shown that this bacterium has gained the ability to proliferate within an infected macrophage by inhibiting the fusion of autophagosomes to lysosomes (Armstrong and Heart, 1971; Clemens and Horwitz, 1995). Recently, the *M.tb eis* gene was shown to regulate the host autophagic response (Shin *et al*., 2010b) and a more recent investigation by Seto and co-workers showed that *M.tb* corrupts the function of the host's coronin 1a protein, a protein that localises to phagosomes containing *mycobacteria*, allowing for the survival of the bacteria within macrophages (Seto *et al*., 2012).

Taken together these studies provide compelling evidence for the role of autophagy in immunity to *M.tb* and given the importance of autophagy to destroy mycobacteria, it is of utmost importance to gain a clear understanding of the natural range of autophagic responses elicited by different mycobacteria. This was partially addressed in a study by Zullo and Lee (2012) which showed that autophagy induction by mycobacteria differs in magnitude and varies by species. In contrast to *Mycobacterium smegmatis* and *Mycobacterium fortuitum* that induces strong autophagic responses; *M.tb* H37Ra, H37Rv and *Mycobacterium bovis* BCG were shown to be weak inducers of autophagy (Zullo and Lee, 2012). However, the question still remains as to whether there are differences within strains. This is an extremely important question to address since evidence suggests that some mycobacterial strains are more virulent than others (Nicol and Wilkinson, 2008). It is therefore reasonable to hypothesize that since there are interspecies differences in mycobacteria with respect to induction and magnitude of the autophagic response that this may also be the case within species, contributing to the understanding of the observed differences in virulence between *M.tb* strains. One can further hypothesize that genes involved in the autophagy pathway are plausible TB susceptibility genes.

The present study was therefore a pilot study investigating the effects on the expression of autophagy associated genes following infection of macrophages with different mycobacterial strains. Additionally, this study hypothesized that highly up- or down-regulated genes in macrophages following *M.tb* infection are good candidate genes for TB susceptibility.

Differentiated THP-1 cells, a monocyte-derived cell line that can be differentiated to macrophages, was infected with seven *M.tb* strains (table 2.1.) and the expression levels of 84 autophagy associated genes were determined using the predesigned RT^2 Profiler human autophagy PCR array (Qiagen, Hamburg, Germany) and the accompanying RT^2 Profiler PCR Array Data Analysis version 3.5 software package.

As expected, several of the autophagy associated genes were differentially expressed in response to *M.tb* infection. Therefore, in order to identify the most likely TB susceptibility genes from the array, only genes that were more than 10-fold differentially expressed were used in an association study of TB susceptibility in a cohort of SAC TB patients and control individuals.

Thirty genes were selected for the case-control association study. Using genotyping data for 382 TB cases and 389 control individuals genotyped on the Illumina MEGA® array as part of a larger study, the present study found no association between any of the 30 selected autophagy associated genes and TB susceptibility in the SAC population after correcting for multiple tests. However, several nominal associations were identified.

4.1 GENE EXPRESSION ANALYSIS

4.1.1 Selection of *M.tb* strains

The Division of Molecular Biology and Human Genetics has a large collection of *M.tb* strains collected from all over South Africa. All of these strains have been strain typed using Spoligotyping (spacer oligonucleotide typing) and *IS6110* DNA fingerprinting.

The major lineages of *M.tb* is illustrated in figure 1.4, and are grouped and named according to their graphical distribution. Lineage 1 is an Indo-Oceanic lineage; Lineage 2 is from East-Asia and includes the Beijing strains; Lineage 3 includes the CAS strains; Lineage 4 is from Euro-America and includes H37Rv; Lineage 5 is also known as West Africa 1 and Lineage 6 is West Africa 2 (Sarkar et al., 2012).

The LAM and Typical Beijing strains were shown to be responsible for the majority of TB cases in Cape Town (Sarkar et al., 2012; Institute of Medicine (US) Forum on Drug Discovery and Science, 2011). Moreover, these two strains together with the LCC strains are responsible for most of the MDR-TB cases in the Western Cape Province (Institute of Medicine (US) Forum on Drug Discovery and Science, 2011). The CAS/Kili strains (Lineage 3) are related to the Beijing strains (Lineage 2) by genome-based phylogeny, but predominantly occurs in the Indian subcontinent and areas of East Africa that have been exposed to Indian migration (Sarkar et al., 2012). Lineage 3 also occurs in the United Kingdom among Indian populations but is uncommon in Cape Town (Sarkar et al., 2012). Lineage 4 consist of Haarlem, Latin American Mediterranean (LAM) and H37Rv and are found throughout Europe, America, certain areas of Africa and the Middle East (Sarkar et al., 2012).

Sekati and Nchabeleng (Sekati et al., 2015) conducted an epidemiological study of 104 *M.tb* isolates from four South African provinces, the Eastern Cape, KwaZulu-Natal, Mpumalanga and North West and showed, that similar to the Cape Town, the LAM and Beijing strains were the dominant strains. The LAM lineage was represented by up to 35%, the Beijing lineage by up to 23% and the CAS/Kili strain was found in low proportions (1%) (Sekati et al., 2015).

A study by Hanekom and co-workers also showed that the LCC clade, LAM, Haarlem and Beijing strains are predominant clades in Cape Town (Hanekom et al., 2007), while Haarlem and Beijing strains are also global clades (van der Spuy et al., 2009). The LCC clade (van der Spuy et al., 2009; Ioerger et al., 2010), LAM and Haarlem strains are prevalent and incidence rates are stable, while the incidence of the Beijing strain rapidly increased possibly due to drug resistance (van der Spuy et al., 2009).

Based on these epidemiological studies, representative strains from each of the above-mentioned strain families were chosen as they were shown to be responsible for much of the TB infections in South Africa and in particular, the Western Cape province.

4.1.2 Selection of a cell line

In the present study the human THP-1 cell line was used. This cell line is a monocytic cell line isolated from peripheral blood of patients suffering from acute monocytic leucemia and is commonly used for studying immune responses (Chanput et al., 2014). This cell line has also been used in previous studies investigating differential gene expression (Maeß et al., 2010; Chen et al., 2013).

These cells are quite versatile and have a doubling time of approximately 35 to 50 hours under ideal growth conditions. This means that these cells are able to quadruple within three and a half days which is a much faster rate compared to PBMC-derived monocytes. This makes them ideal for *in vitro* studies. Furthermore, the THP-1 cell line is an immortalised cell line that can be cultured *in vitro* up to passage 25 without having any effect on cell sensitivity and activity (Chanput et al., 2014). It should also be noted that PBMC-derived monocytes require stimulation with inflammatory mediators such as IL-1β, TNFα or lipopolysaccharide to prevent cellular apoptosis (Mangan et al., 1991; Mangan and Wahl, 1991), while this is not the case for THP-1 cells.

Moreover, THP-1 cells are genetically homogeneous and therefore the variability in cell phenotype is minimised which facilitates the reproducibility of experimental results. For these reasons, the THP-1 cell line was an ideal choice of cell type for the present study.

THP-1 cells can be differentiated into a macrophage-like phenotype by treating them with either phorbol-12-mysistrate acetate (PMA), 1α 25-dihydroxyvitamin D3 (vD3) or macrophage colony-stimulating factor (MCSF). Treatment of THP-1 cells with vD3 (100nm) for three days was shown to result in macrophages that were less to PBMC-derived macrophages in terms of their cytokine profile and phagocytic activity when compared to THP-1 cells differentiated for three days using PMA. (Chanput et al., 2014). Therefore, in the present study, PMA stimulation for three days was used.

4.1.3 Differential gene expression

Analysis of differential gene expression following infection of THP-1 cells with different *M.tb* strains identified several genes that were more than two-fold up- or down-regulated (Appendix III). Of particular interest in this study were genes that were highly differentially expressed following infection as we hypothesized that highly regulated genes are good candidate TB susceptibility genes. For this reason, in this pilot study, more emphasis was placed on genes that were differentially expressed more than 10-fold.

The 30 genes with the biggest fold change values were selected for the case-control association. The majority of the highly up-regulated genes were found to be co-regulators of autophagy and apoptosis.

It has long been established that apoptosis plays a significant role in the host immune response to infection (Khan et al., 2016). In the case of *M.tb* infection, apoptosis disrupts the bacterium's intracellular protective niche, forcing it to find a different habitat.

Mycobacterium tuberculosis induces apoptosis via the classical extrinsic pathway. When the bacillus encounters innate immune cells such as macrophages and dendritic cells (DCs), it induces the release of TNFα, resulting in the induction of apoptosis which limits the replication of *M.tb* by sequestering it to an apoptotic vesicle (Khan et al., 2016). In the present study, with the exception of the LCC strain, all other *M.tb* strains tested induced the up-regulation of TNFα.

Interestingly, several reports have suggested that virulent *M.tb* induces necrosis in an effort to avoid host immune responses whereas attenuated strains are associated with apoptosis (Chen et al., 2006; Divangahi et al., 2009). This phenomenon was shown to occur regardless of the amount of TNFα. Further investigations found that cells infected with virulent *M.tb* strains secrete more IL-10 thereby inducing the release of the soluble form of TNFR-2 which complexes with TNFα resulting in the down-regulation of TNFα-induced apoptosis (Balcewicz-Sablinska et al., 1998).

The effect on autophagy is dependent on the level of function lost, any autophagy-independent effects on the specific genes and in which stage the genes functions of the autophagy process (Cecconi and Levine, 2008). Atg genes also play a role in the cytoplasm to vacuole (Cvt) pathway and/or pexophagy (Meijer et al., 2007). The genes included in the array are not exclusively involved in the autophagy pathway, although the included genes are key genes in regulating this pathway. Many of the genes are also involved in another cell death cycle, apoptosis, while other genes (CXCR4) support the entry of HIV into cells and is highly up-regulated in breast cancer cells ("Look for Gene | Autophagy database").

4.1.4 Genes involved in autophagy induction and autophagic vacuole formation

Several genes involved in the formation of the autophagic vacuole were found to be down-regulated following infection with each of the *M.tb* strains in the present study (figure 3.12). This indicates that autophagic vacuole formation is impaired following infection of THP-1-derived macrophages which is consistent with previous investigations showing impaired autophagosomal formation following *M.tb* infection. Seto and co-workers (2012) showed that *M.tb* recruits the human protein Coronin-1 (Coro1a) to *M.tb* to inhibit autophagosomal formation and that depletion of Coro1a promoted autophagosomal formation. This inhibition of autophagosomal facilitates the survival of *M.tb* in macrophages.

Furthermore, the mycobacterial Eis protein has also been shown to inhibit autophagic vacuole formation. Shin and colleagues (2010a) found that infection of bone marrow-derived macrophages (BMDM) with an *eis*-deletion mutant H37Rv (*M.tb-Δeis*) resulted in the increased accumulation of large autophagic vacuoles and increased autophagosome formation compared to BMDM infected with wild type H37Rv. In fact, there was no difference in autophagic vacuole formation in BMDM infected with H37Rv compared to uninfected cells (Shin et al., 2010a).

Additionally, defects in autophagy induction has been correlated with poor TB outcomes (Li et al., 2016). Li and co-workers (2016) demonstrated that infection of THP-1 cells with

different *M.tb* clinical isolates resulted in varying degrees of autophagy induction and that clinical isolates that impair autophagy induction were associated with poorer prognosis. In our study we lack the clinical data and thus cannot make comparisons to the findings of Li et al.

Dendritic cells, however, respond differently to *M.tb* infection compared to macrophages. In DCs, proliferation of *M.tb* is restricted, but not in macrophages (Tailleux et al., 2003). In contrast to macrophages, infection of DCs with *M.tb* leads to the selective autophagosomal formation (Tailleux et al., 2003).

4.1.5 Genes involved in the fusion of autophagosomes to lysosomes

Infection of THP-1 macrophages with each of the investigated strains resulted in the down-regulation of *LAMP1*. Lysosome associated membrane protein 1 is a major component of the lysosomal membrane and together with *LAMP2* it contributes to roughly 50% of the lysosomal membrane proteins. It is a type I transmembrane protein with a large luminal domain, a transmembrane domain and a C-terminal cytoplasmic tail (Eskelinen, 2006). One of the major definitions of a lysosomal compartment is the presence of *LAMP* proteins and *LAMP1* is considered a marker of lysosomal biogenesis. Previous studies have suggested that *LAMP* proteins are crucial for the lysosomal integrity and to protect the lysosomal membrane against the actions of hydrolytic enzymes within the lysosome (Eskelinen 2003). However, degradation of *LAMP1* and *LAMP2* by depletion of N-glycans with endoglycoside H causes no changes in lysosomal integrity suggesting alternative *LAMP* functions (Kundra and Kornfeld, 1999). Moreover, in *LAMP2* knockout studies the mice demise on average at about 20-40 days postnatal (Andrejewski et al., 1999; Eskelinen, 2006). In contrast deletion of *LAMP1* resulted in mice that were viable and fertile with a less severe phenotype. These data may suggest that while *LAMP1* is an important lysosomal marker, it may not play such a crucial role in lysosomal integrity. Subsequently, Huynh and colleagues (2007) showed that *LAMP1* and *LAMP2* are required for the fusion of lysosomes with phagosomes. LAMP2 was found to be up-regulated between 1.7 and 2.7 fold in *LAMP1* knockout mice, suggesting that

elevation of the structurally related *LAMP2* gene can efficiently compensate for the loss of *LAMP1* and that *LAMP1* and *-2* have overlapping functions (Andrejewski et al., 1999).

In the present study, given that all of the tested strains induce a decrease in *LAMP1* expression, it may be speculated that *M.tb* is able to block autophagosomal fusion with lysosomes by down-regulating *LAMP1* expression. It should be noted, however that in *LAMP1* knockout mice, *LAMP2* expression is increased in order to compensate for the loss of *LAMP1* (Andrejewski et al., 1999). Here, we did not evaluate the expression of *LAMP2* as this gene was not represented on the array. Therefore, it may be likely that the down-regulation of *LAMP1* following *M.tb* infection is compensated for by the up-regulation of *LAMP2*, but this needs to be investigated in future studies. It is important to note that to our knowledge no previous studies have investigated the effects of *LAMP1* deficiency in lung tissue and that this is the first study to look at *LAMP1* levels after *M.tb* infection *in vitro*.

GABA receptor-associated protein (*GABARAP*) is an ubiquitin-like protein and is a homologue of the yeast *Atg8* which plays a role in autophagosome biogenesis and recruitment of cargo to autophagosomes. The *Atg8* family of proteins consists of six proteins that can be broadly catogorised into two large subfamilies, the LC3s and the *GAPRAPs*. While the roles of LC3 proteins in autophagy has been extensively investigated, there is a paucity of studies into the functions of *GAPRAPs*.

Wang and colleagues showed that unlike LC3s, *GABARAPs* bind phosphatidylinositol 4-kinase Iiα (PI4KIIα), which is a lipid kinase involved in the generation of phosphatidylinositol 4-phosphate (PI4K). When either *GABARAP* or PI4KIIα is deleted it causes a decrease in autophagy flux by blocking autophagolysosomal fusion resulting in an accumulation of abnormally large autophagosomes (Wang et al., 2015). The defects are observed in the autophagosomes ameliorated following the addition of exogenous (PI4K) which suggests that, *GABARAPs* governs the fusion of autphagosomes to lysosomes in a PI4KIIα-mediated *in situ* generation of PI4K (Wang et al., 2015).

In the present study, infection of THP-1 cells with any of the investigated strains resulted in significant up-regulation of *GABARAP*. This is consistent with the notion that *M.tb* infection causes impairments in autophagolysosomal fusion (section 1.2.3.3.2)

Damage-regulated autophagy modulator (*DRAM*) is a lysosomal protein which links p53 to autophagy and functions to regulate the lysosome membrane permeabilisation and programmed cell death of cells infected with a pathogen. The inhibition or depletion of *DRAM* or p53 prevents lysosome membrane permeabilisation and results in the inhibition of autophagy in infected cells (Laforge et al., 2013). In the present study, three of the tested strains (Atypical Beijing, Cas/Kili, and H37Rv) were able to up-regulate *DRAM1*. The representatives of these strain families that were investigated in this study, are considered weakly virulent so it is tempting to speculate that the up-regulation of *DRAM1* in these strains may promote autophagolysosomal fusion. Notably, however, while *DRAM* plays a crucial role in autophagy, Crighton et al., (2006) showed that the overexpression of *DRAM* is not sufficient to induce autophagy.

Niemann-Pick type C1 (*NPC1*) encodes the membrane spanning protein, NCP1 which functions in late endosomes to enhance lipid sorting and vesicular trafficking (Raben et al., 2009). Cells with mutations in either *NPC1* or *NPC2* results in accumulation of un-esterified cholesterol in late endosomal/lysosomal organelles (Frolov et al., 2003).

NPC1 is primarily involved in the Niemann-Pick disease which is a fatal, autosomal recessive lipid storage disorder and is characterised by accumulation of cholesterol in the spleen, liver and central nervous system (Frolov et al., 2003). It is believed that the accumulation of cholesterol in the endosomal/lysosomal system impairs the fusion of autophagosomes with lysosomes and that the accumulated cholesterol can impair cell function by trapping the autophagic machinery and other proteins in the late compartments of the endocytic pathway (Liao et al., 2007).

In our study, three of the seven strains down-regulated all four genes, namely LAM, LCC and Haarlem 3, which may suggests that of the seven strains tested, and these three

most successfully inhibit the autophagolysosome fusion and ensure the survival of the mycobacterium. The remaining four strains successfully down-regulated at least two of the four genes, (in most cases *LAMP1* and *GABARAP*) involved in the fusion of autophagolysosome.

4.1.6 Typical Beijing vs. Atypical Beijing

The Beijing genotype strains of *M.tb* are becoming widespread. This may be associated with antibiotic resistance or through the manipulation of an impaired host immune system (Hanekom et al., 2011; Parwati et al., 2010). It is subdivided into two broad sublineages, the typical (modern) Beijing and the atypical (ancient) Beijing based on IS6110 DNA fingerprinting (Ribeiro et al., 2014) .

The typical Beijing strain represents between 65% and 95% of circulating Beijing strains in most regions, including South Africa (Strauss et al., 2008). There is a degree of genetic conservation within typical Beijing strains which suggests that they may have a selective advantage over their atypical counterparts (van Laarhoven et al., 2013). It is thought that the reason for the success of the typical Beijing strains is that they are able to elicit a different or attenuated host immune response compared to the atypical Beijing strains. Typical Beijing strains induce significantly less IL-1β and moderately higher IL-1Ra compared to the atypical strains (van Laarhoven et al., 2013). Since IL-1Ra is an IL-1β antagonist, this further depletes the pool of available IL-1β during infection.

Additionally, typical Beijing strains induces significantly less IFN-γ following infection compared to atypical Beijing strains (van Laarhoven et al., 2013). Consistent with this is our observation of a 4-fold decrease in IFN-γ in THP-1 cells infected with the typical Beijing strain compared to the atypical Beijing strain (Table 3.9, Figure 3.9). Typical Beijing strains also induces moderate TNFα expression compared to atypical Beijing strains (van Laarhoven et al., 2013), again consistent with the findings of the present study. The lower IFN-γ response by typical Beijing strains coupled with the production of less IL-1β and their moderate up-regulation of IL-1Ra which leads to a stunted immune response may explain their increased spread globally.

Interestingly, we also noted a significant increase in the expression of *ATG5* and a significant decrease in the expression of *RPS6KB1* in THP-1 cells infected with the typical Beijing strain compared to THP-1 cells infected with the atypical Beijing strain (fig 3.9). The involvement of *RPSK6KB1*, which encodes the Ribosomal protein S6 kinase, still remains controversial. While a number of studies have suggested that up-regulation of *RPSK6KB1* leads to a decrease in autophagy induction (Blommaart et al., 1995; Lee et al., 2007; Yorimitsu et al., 2007), several more have shown that up-regulation of RPSK6KB1 leads to an increase in autophagy induction (Armour et al., 2009; Hać et al., 2015; Scott et al., 2004; Shin et al., 2013).

The protein encoded by *ATG5*, plays a critical role in the formation of autophagosomal membrane formation. It forms part of the Atg12-Atg5-Atg16 complex which acts as an E3 ubiquitin ligase for the conjugation of LC3 and specifies the site of LC3-PE production (section 1.2.3.3.). Given that ATG5 plays such a crucial role in autophagosomal formation, one could speculate that the increase expression of ATG5 observed in typical Beijing strain infected cells indicates that the atypical Beijing strain inhibits autophagosomal membrane formation more efficiently than the typical Beijing strain. It is therefore likely that while typical Beijing strains do not effectively inhibit autphagosomal membrane formation once autophagy is induced, they are more effective at inhibiting autophagy induction to begin with based on their ability to down-regulate IFN-γ expression.

4.1.7 Highly virulent strain vs. less virulent strain

To investigate the differences in the autophagy pathway as it pertains to virulence, differential gene expression following infection with a highly virulent Beijing strain was compared to a less virulent CAS/Kili strain (fig 3.10). The heat map indicates an overall down-regulation of the autophagy-associated genes in cells infected with the Beijing strain compared to the CAS/Kili strain, which indicates the more virulent Typical Beijing strain exerts a bigger influence on the autophagy pathway compared to the less virulent CAS/Kili strain.

Three genes were highly up-regulated when Typical Beijing was compared to CAS/Kili, which serves as the control in this case. Two of the genes, *ATG5* and *ATG9B*, are involved in the vacuole formation and *CDKN2A* co-regulates autophagy. *DRAM1* and *TNF* were highly down-regulated and are both co-regulators of autophagy, while *DRAM1* further influences the autophagolysosome fusion. To summarise, the Typical Beijing strain is more equipped to inhibit the autophagy pathway than the CAS/Kili strain. Even though the strain enables the formation of the autophagic vacuole, it is successful in halting the autophagolysosome fusion, enabling survival of the pathogen.

4.1.8 The top 30 genes differentially expressed.

The importance of ATGs for the formation of autophagosomes was originally identified in yeast and they are regulated by conserved nutrient and energy-dependent signaling cascades which involves the mTOR pathway and AMP-activated protein kinase (*AMPK*) (Proikas-Cezanne et al., 2015). *AMPK* and mTOR control *ULK1* and *ULK2*.

Autophagy is enabled in nutrient limiting conditions, which is activated by *AMPK* in low cellular levels of ATP either indirectly by mTOR complex 1 (mTORC1) inactivation or directly by *ULK1* phosphorylation (Proikas-Cezanne et al., 2015; Fritzen et al., 2016). Phosphatidylinositol 3-phosphate (PtdIns3*P*) production by beclin 1 phosphorylation is stimulated after *ULK1* activation and *ATG13* and *FIP200* phosphorylation, and this leads to the activation of phosphatidylinositol 3-kinase class III (*PI3KC3*). *PI3KC3* together with beclin 1, p150 (aka *PIK3R4*) and *ATG14L* relocates to the initiation site for the formation of autophagosomes, with *ATG14L* being vital in directing the *PI3KC3* complex to the ER (endoplasmic reticulum). At the ER the *ULK* complex is stabilized by the *PI3KC3* complex.

For the phagophore expansion to occur, *ATG12* and *LC3* which are autophagosomal ubiquitin-like conjugation systems genes, are needed and are known as *LC3* lipidation. Before *LC3* lipidation occurs and downstream of PtdIns3*P* production, the *ATG16L1* complex is recruited to the initiation site for the formation of autophagosomes. The *ATG16L1* complex consists of *ATG12* conjugated to *ATG5* and associated with *ATG16L*

(ATG12-ATG5-ATG16L). This complex aids in mediating *LC3* lipidation. The functions of *LC3* as a membrane protein of early and mature autophagosomes are to assist hemifusion procedures during the expansion and closure of the phagophore; *LC3* enables specific cargo recognition by binding to proteins that contains the *LC3-interacting region (LIR*); and it enables adaptor protein docking (Proikas-Cezanne et al., 2015).

4.1.9 The various ways the autophagy pathway is manipulated by the different *M.tb* strains

The seven *M.tb* lineages can be subdivided into modern and ancient (section 1.1.2.3; fig 1.4). The modern lineages comprise of lineage 2, 3 and 4, while the ancient lineages are 1, 5 and 6 and these three lineages are also known as *Mycobacterium Africanum* lineages. Lineage 7 is known as the intermediate lineage and occurs in Ethiopia (Gagneux, 2012). The strains of lineage 2, including the Beijing family, are known to replicate quickly in the human cell culture model and in the lungs of infected mice. Various human and animals studies have proposed that the enhanced virulence of this lineage can be due to its ability to lower levels of protective Th1 cytokines, including *TNF, IL-12* and *IFN-γ* (Sarkar et al., 2012). Genome-based phylogeny confirms that lineage 3 is related to lineage 2, but is considerably more geographically restricted and mostly occurs in the Indian subcontinent and in regions of East Africa and the United Kingdom with high numbers of Indian migration. This strain is uncommon in Cape Town. The CAS family belongs to lineage 3 and are characterised as “slow growers” (Sarkar et al., 2012). Lineage 4 includes the laboratory strain H37Rv, LAM and Haarlem and is found throughout America, Europe and parts of the Middle East and Africa. H37Rv is known to grow well in monocyte derived macrophages and exhibit similar induced cytokine patterns as Haarlem and LAM strains in human macrophages (Sarkar et al., 2012).

When all the results are combined and analysed, it is possible to determine where the individual strains primarily influence the autophagy pathway (fig 4.1). The LAM and the CAS/Kili strains elicited the weakest IFN-γ response in the THP-1 cells which indicates that of the seven strains tested, these two were the weakest inducers of autophagy. All of the strains except the typical Beijing strain significantly down-regulated the expression

of *ATGs* involved in autophagosomal membrane formation. Disruption of membrane formation leads to an inability of the cell to form intact autophagosomal vacuoles. These vacuoles are important for the isolation of *M.tb* from the rest of the cell and maintaining the internal pressure and acidic internal pH of the cell (Wada, 2013). Boller et al. (1975) and Jezbera et al. (2005) proposed that the vacuole has an important role in autophagy to maintain the balance between biosynthesis and degradation, while aiding in lysis and recycling of misfolded proteins. It also participates in the destruction and containment of invading mycobacteria.

Additionally, as expected, all the strains were down-regulated genes involved in the fusion of autophagosomes to lysosomes, however, the Haarlem 3 strain, the LAM 1 strain and the typical Beijing strains were most effective at accomplishing this. The LAM strain influences protein transport, protein targeting to the membrane and vacuole and the protease activity. Imbalances in protein homeostasis are known to cause an increasing number of diseases, which shows the importance of proper cellular protein quality control (Kim et al., 2013).

All seven strains are also co-regulators of the autophagy pathway and can influence the pathway directly (as mentioned above) or indirectly through genes that are not directly located in the pathway but form part of the process, which leads to increased survival and proliferation of *M.tb*.

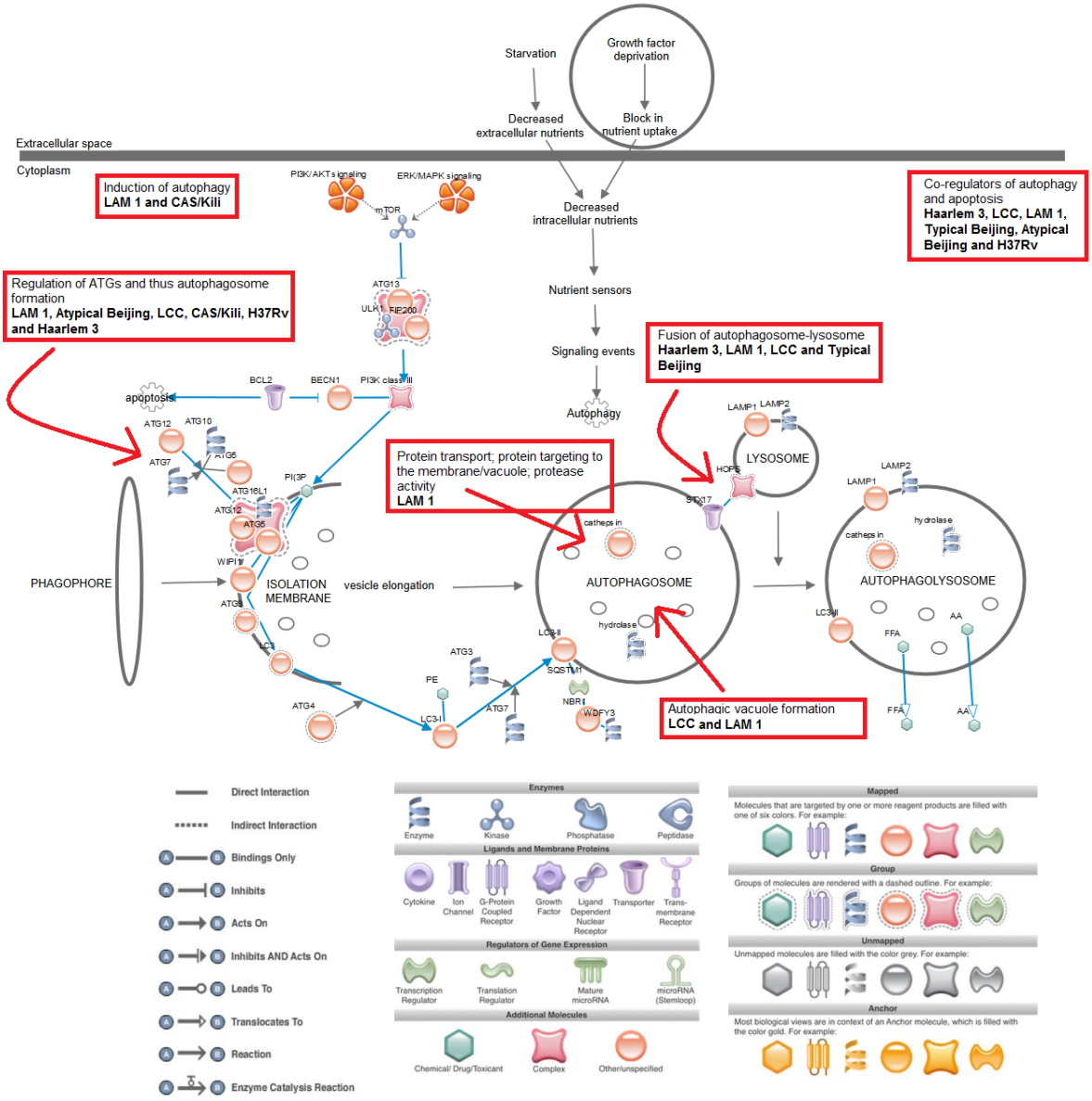

Figure 4.1. Summary of the autophagy pathway. The genes that are responsible for every step within the autophagy pathway, as well as the step(s) they are responsible for, are indicated on the figure. The keys to the figure are located in the blocks, the first block describes the relationship, the second indicates the molecules and the third the molecule colours (IPA® analysis website ("autophagy Pathway - Target Explorer," 2016)).

Our conclusions are consistent with findings from other studies showing that *M.tb*-containing autophagosomes can successfully evade the fusion with lysosomes, therefore halting autophagosomal maturation during infection with *M.tb* which enables survival and

proliferation of the bacteria (Vergne et al., 2006; Philips, 2008). *Mycobacterium tuberculosis* is able to halt the maturation and fusion process of the autophagosome through interfering with Ca^{2+} signaling as well as trafficking of the Rab family (section 4.2.3.6). Once the pathogens are in the autophagosome, they secrete tyrosine phosphate to decrease the phagosomal level of PtdIns3*P* or the pathogens can inhibit the host proteins responsible for regulating the vacuole sorting, and both these processes leads to the impaired fusion of the autophagolysosome (Shui et al., 2011).

4.2 GENOTYPING AND COHORT STUDY

It has been established that host genetic factors are vital in the determination of the host's susceptibility to the pathogen (Newport et al., 1996; Altare et al., 1998; Lu et al., 2016). Given the important role of autophagy in the host immune response to TB, genetic variation in genes encoding components of the autophagy pathway may have an effect on the host immune response to TB. In fact, mice with defective autophagy associated gene expression are more susceptible to TB infection (Divangahi et al., 2009; J.D. MacMicking et al., 2003) and human mononuclear cells with varying polymorphic alleles of *P2X7* receptor was shown to have impaired ability to control *M.tb* growth (Fernando et al., 2005).

Furthermore, polymorphisms in immunity-related GTPase family M (*IRGM*) has been associated with TB susceptibility in a number of independent studies. *IRGM* encodes a GTP-binding protein that induces autophagy. Inhibition of *IRGM* expression causes impaired autophagy resulting in the prolonged survival of *M.bovis* BCG in macrophages (Singh et al., 2006). An increased risk of TB has been associated with variants in the *IRGM* promoter region. The SNP rs10065172 was determined as a risk allele for susceptibility to TB in African-Americans and Koreans population (King et al., 2011; Lu et al., 2016). Additionally, an increased susceptibility to TB was found in a Han Chinese population. Individuals carrying the C allele of rs10065172, those carrying the T allele of rs10051924 and those carrying the T allele of rs13361189 of *IRGM* showed increased susceptibility to TB (Lu et al., 2016). On the other hand rs10065172 was significantly

associated with a decreased susceptibility to TB in a Korean population (Song et al., 2014). No associations was however observed in a Ghanaian cohort for SNPs in *IRGM* (Intemann et al., 2009). *P2X7*, another autophagy associated gene has also been associated with TB susceptibility. Polymorphisms in *P2X7* was found to have a protective affect against TB susceptibility in a case-control cohort of 300 Gambian TB cases and 160 matched controls were tested (Li et al., 2002). In Southeast Asians Fernando et al, (2007) reported that polymorphisms in *P2X7* increased susceptibility to extra-pulmonary TB, and that this defect is associated with macrophages having a reduced ability to kill *M.tb*.

4.2.1 Cohort results

Following the analysis of SNPs in each of the 30 top differentially expressed autophagy associated genes in the present study genotyped using the Illumina MEGA® array in 382 TB cases and 389 control individuals, no statistically significant association was found following correction for multiple testing. Similarly, in a study of 14 autophagy associated genes in a cohort of 1 022 Indonesian TB patients and 952 matched controls, no association was found (Songane et al., 2012). Admittedly, only one gene, *WIPI1*, which was genotyped in our study was also genotyped in the study by Songane and colleagues (2012), but it is interesting to note that neither study provided evidence for an association with autophagy associated genes.

One reason for a lack of significant association observed in the present study is the limited sample size. Here, we had genotyping data for 382 TB cases and 389 control individuals. We calculated 100% statistical power using the CaTS power calculator to determine an odds ratio of 2 given the allele frequency of at least 0.158 for the nominal associated SNPs, with a disease prevalence of 1% as calculated for Cape Town by den Boon et al., (2007).

It is also possible that autophagy itself does not play a major role in the immune response to TB. Kimmey and co-workers (2015) showed contrary to dogma that the autophagic capacity does not correlate with *M.tb* infection. In their investigation they focused on the

role of several autophagy factors including *ATG5, ULK1* and *ULK2*, *p62*, and *ATG4b* in TB infection in knock out mouse models and found that only *ATG5* knockout mice were more vulnerable to TB infection. Additionally, they showed that ATG5 plays an autophagy-independent role during *M.tb* infection. They conclude that loss of genes that are essential in the canonical autophagy pathway do not influence susceptibility to TB and therefore autophagy itself may not be an important mediator of TB immunity. Alternatively, some of the uninvestigated genes, with small gene expression differences could possibly contain important SNPs associated with TB susceptibility.

These results do not mean that the SNPs are not important or that they are not vital in our understanding of the mechanisms of *M.tb*, it simply means that these SNPs remain to be investigated in other populations where their significance may change due to the different allele frequencies.

4.3 LIMITATIONS AND FUTURE STUDIES

The first limitation would be using THP-1 cells. The ideal cell type to have used for all experiments would be alveolar cells. The only way to obtain alveolar cells is through a bronchoalveolar lavage (BAL) which is a very invasive medical procedure typically performed in patients where a lung disease is suspected. A bronchoscope is passed though the mouth or the nose into the lungs, sterile saline is pumped into a small part of the lung and removed to be analysed. This ensures fluid and cells from bronchioles and lung alveoli are collected for examination. This procedure is unpleasant for patients and through this method only a small number of cells are collected. For this project healthy individuals are required and large amounts of cells are needed to extract a sufficient amount of RNA for optimal gene expression. Thus in future studies ethics will need to be obtained to perform BAL in healthy individuals various times in order to do the experiments in the most ideal cells.

The second limitation was our RIN values. RNA Integrity Numbers are representative of the eukaryote total RNA sample and includes the absence or presence of degraded

products. These values are also independent of the instrument, the analyst and the sample concentration. RIN values should preferably exceed 7, meaning that the integrity of RNA is good enough to use in the experiments planned and the RNA is not entirely degraded. The RIN value is not a complete predictor of the results an experiment will yield, in some techniques a high RIN value is not necessary for success, whereas in other cases it is.

We obtained lower value RIN numbers for some of the samples and speculate that this is due to our starting amount of cells. Due to working in a BSL-3 laboratory the maximum amount of liquid culture is limited to 15mL and 6-well plates. This limitation prohibited the ability to obtain high RNA concentrations. The RNeasy® Mini Kit (Qiagen, Hilden, Germany) protocol required a maximum of 1 x 10^7 cells and that is what we seeded, while the Qiagen RT^2 first strand kit (Qiagen, Hilden, Germany) required a minimum of 25ng RNA and we converted roughly 100ng RNA per reaction. Thus enough RNA was obtained to convert to cDNA according to the Qiagen RT^2 first strand kit protocol.

We hypothesis that once we increase our initial cell number we are extracting RNA from, that our RIN values and RNA concentration will increase. In future studies we will pool wells together for better RNA extraction from larger amounts of cells to ensure more optimal values. The low RNA concentration values affected our gene expression work. We compromised for the fluctuating concentration values by standardising the concentration for each sample that was added to the array to ensure the correct results were obtained.

Thirdly, we did not validate our findings from our gene expression results with western blots. Western blots is an excellent and reliable test, since it is able to determine the relative protein expression. RNA degradation can occur for various reasons, including inadequate sample handling, continued storage and suboptimal storage conditions (Vermeulen et al., 2011). The western blots could confirm that our results are correct on a protein level. Western blots were not performed, because antibodies did not arrive in

time due to unforeseen problems with the import permit. Future work will entail doing western blots to confirm our gene expression results.

Since we did not detect a significant association between any of the 30 genes evaluated and TB susceptibility in the present study, future studies will include the remaining 54 genes on the RT^2 Profiler® autophagy array. Additionally, we will perform case control association studies for previously investigated autophagy associated genes (Fernando et al., 2007; Intemann et al., 2009; Li et al., 2002; Songane et al., 2012). However, increasing the number of genes tested will also increase the number of SNPs evaluated. This will require more stringent Bonferroni correction. This, together with the small sample size could influence the power calculations and increase the chance of overlooking genes with small to moderate effects.

4.4 CONCLUDING REMARKS

Mycobacterium tuberculosis infection remains a global burden which causes approximately 1.8 million death yearly(World Health Organisation, 2015). It has been determined that one of the factors that ensures the survival of the bacterium, is its pathogenicity which enables survival within the host macrophages (Hingley-Wilson et al., 2003). A wide range of factors has been associated to be involved in the survival of *M.tb* within the host and lately Zullo and Lee (2012) demonstrated that the mammalian autophagy pathway is one of the major determinants in the course of infection. The virulence of the *M.tb* strains has further been shown to have the ability to inhibit the autophagy pathway (Kumar et al., 2010). Therefore it is of utmost importance to gain a clear understanding of the autophagic responses elicited by the different mycobacteria in order to overcome autophagy.

We hypothesized that since there are interspecies differences in mycobacteria with respect to induction and magnitude of the autophagic response that this may also be the case within species, contributing to the understanding of the observed differences in virulence between *M.tb* strains. We were able to show significant differences in the

expression of several autophagy associated genes following infection with different *M.tb* strains suggesting that different *M.tb* strains elicits different autophagic responses in the host.

Several nominal associations were found between variants in the top 30 differentially expressed autophagy associated genes and TB susceptibility. However, after correcting for multiple testing, none of these remained significant. This could be due to inadequate sample size or the fact that autophagy associated genes play very minor roles, if any, in TB susceptibility in our cohort.

This is, to the best of our knowledge, the first *in vitro* study to investigate differential gene expression of autophagy associated genes following infection with varying *M.tb* strains. We envisage that future studies would investigate many more *M.tb* strains using RNA sequencing to investigate global gene expression differences elicited by different *M.tb* strains. We do however, believe that this study provides novel candidate genes that can be targeted in future TB pharmacotherapy.

To conclude, this study aids in our understanding of how *M.tb* (especially strains responsible for the epidemic in South Africa) manages to overcome the host immune system, more specifically which genes each specific strain investigated in our study uses to its advantage. This can aid in identifying novel mechanisms of drug resistance and could lead to better therapeutics for the treatment of this devastating disease.

REFERENCE LIST

Aguilar L, D., Hanekom, M., Mata, D., Gey van Pittius, N.C., van Helden, P.D., Warren, R.M., Hernandez-Pando, R., 2010. Mycobacterium tuberculosis strains with the Beijing genotype demonstrate variability in virulence associated with transmission. Tuberculosis 90, 319–325. doi:10.1016/j.tube.2010.08.004

Altare, F., Durandy, A., Lammas, D., Emile, J.F., Lamhamedi, S., Le Deist, F., Drysdale, P., Jouanguy, E., Döffinger, R., Bernaudin, F., Jeppsson, O., Gollob, J.A., Meinl, E., Segal, A.W., Fischer, A., Kumararatne, D., Casanova, J.L., 1998. Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. Science 280, 1432–1435.

Andrejewski, N., Punnonen, E.L., Guhde, G., Tanaka, Y., Lüllmann-Rauch, R., Hartmann, D., von Figura, K., Saftig, P., 1999. Normal lysosomal morphology and function in LAMP-1-deficient mice. J. Biol. Chem. 274, 12692–12701.

Armour, S.M., Baur, J.A., Hsieh, S.N., Land-Bracha, A., Thomas, S.M., Sinclair, D.A., 2009. Inhibition of mammalian S6 kinase by resveratrol suppresses autophagy. Aging 1, 515–528. doi:10.18632/aging.100056

Armstrong, J.A., Hart, P.D., 1971. RESPONSE OF CULTURED MACROPHAGES TO MYCOBACTERIUM TUBERCULOSIS, WITH OBSERVATIONS ON FUSION OF LYSOSOMES WITH PHAGOSOMES. J. Exp. Med. 134, 713–740.

autophagy Pathway - Target Explorer [WWW Document], 2016. URL https://targetexplorer.ingenuity.com/pathway/ING/ING:e5a7h#!/api/rest/v1/client/searchPathwayNodes?pathwayId=ING:e5a7h&rows=0&facetLimit=5000&responseType=default (accessed 8.11.16).

Baker, L., Brown, T., Maiden, M.C., Drobniewski, F., 2004. Silent nucleotide polymorphisms and a phylogeny for Mycobacterium tuberculosis. Emerg. Infect. Dis. 10, 1568–1577. doi:10.3201/eid1009.040046

Balcewicz-Sablinska, M.K., Keane, J., Kornfeld, H., Remold, H.G., 1998. Pathogenic Mycobacterium tuberculosis evades apoptosis of host macrophages by release of TNF-R2, resulting in inactivation of TNF-alpha. J.Immunol. 161, 2636–2641.

Behar, S.M., Martin, C.J., Booty, M.G., Nishimura, T., Zhao, X., Gan, H.-X., Divangahi, M., Remold, H.G., 2011. Apoptosis is an innate defense function of macrophages against Mycobacterium tuberculosis. Mucosal Immunol. 4, 279–287. doi:10.1038/mi.2011.3

Berry, M.P.R., Blankley, S., Graham, C.M., Bloom, C.I., O'Garra, A., 2013. Systems approaches to studying the immune response in tuberculosis. Curr. Opin. Immunol., Special section: Systems biology and bioinformatics / Immunogenetics and transplantation 25, 579–587. doi:10.1016/j.coi.2013.08.003

Beyer, A., Zu, K., Sikes, M., Osheim, Y., 1996. Role of the individual domains of A/B-type HnRNP proteins in vivo [WWW Document]. Mol. Biol. Cell. URL http://eurekamag.com/research/033/273/033273125.php (accessed 8.18.16).

Blommaart, E.F., Luiken, J.J., Blommaart, P.J., van Woerkom, G.M., Meijer, A.J., 1995. Phosphorylation of ribosomal protein S6 is inhibitory for autophagy in isolated rat hepatocytes. J. Biol. Chem. 270, 2320–2326.

Boehme, C.C., Nicol, M.P., Nabeta, P., Michael, J.S., Gotuzzo, E., Tahirli, R., Gler, M.T., Blakemore, R., Worodria, W., Gray, C., Huang, L., Caceres, T., Mehdiyev, R., Raymond, L., Whitelaw, A., Sagadevan, K., Alexander, H., Albert, H., Cobelens, F., Cox, H., Alland, D., Perkins, M.D., 2011. Feasibility, diagnostic accuracy, and effectiveness of decentralised use of the Xpert MTB/RIF test for diagnosis of tuberculosis and multidrug resistance: a multicentre implementation study. The Lancet 377, 1495–1505. doi:10.1016/S0140-6736(11)60438-8

Boller, T., Dürr, M., Wiemken, A., 1975. Characterization of a specific transport system for arginine in isolated yeast vacuoles. Eur. J. Biochem. FEBS 54, 81–91.

Brighenti, S., Lerm, M., 2012. How Mycobacterium tuberculosis Manipulates Innate and Adaptive Immunity – New Views of an Old Topic, in: Cardona, P.-J. (Ed.), Understanding Tuberculosis - Analyzing the Origin of Mycobacterium Tuberculosis Pathogenicity. InTech.

Briken, V., Porcelli, S.A., Besra, G.S., Kremer, L., 2004. Mycobacterial lipoarabinomannan and related lipoglycans: from biogenesis to modulation of the immune response. Mol. Microbiol. 53, 391–403. doi:10.1111/j.1365-2958.2004.04183.x

Brosch, R., Gordon, S.V., Marmiesse, M., Brodin, P., Buchrieser, C., Eiglmeier, K., Garnier, T., Gutierrez, C., Hewinson, G., Kremer, K., Parsons, L.M., Pym, A.S., Samper, S., van Soolingen, D., Cole, S.T., 2002a. A new evolutionary scenario for the Mycobacterium tuberculosis complex. Proc. Natl. Acad. Sci. U. S. A. 99, 3684–3689. doi:10.1073/pnas.052548299

Brosch, R., Gordon, S.V., Marmiesse, M., Brodin, P., Buchrieser, C., Eiglmeier, K., Garnier, T., Gutierrez, C., Hewinson, G., Kremer, K., Parsons, L.M., Pym, A.S., Samper, S., Van Soolingen, D., Cole, S.T., 2002b. A new evolutionary scenario for the Mycobacterium tuberculosis complex. Proc. Natl. Acad. Sci. U. S. A. 99, 3684–3689. doi:10.1073/pnas.052548299

Brown, L., Wolf, J.M., Prados-Rosales, R., Casadevall, A., 2015. Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. Nat. Rev. Microbiol. 13, 620–630. doi:10.1038/nrmicro3480

Burgner, D., Jamieson, S.E., Blackwell, J.M., 2006. Genetic susceptibility to infectious diseases: big is beautiful, but will bigger be even better? Lancet Infect. Dis. 6, 653–663. doi:10.1016/S1473-3099(06)70601-6

Cain, K.P., McCarthy, K.D., Heilig, C.M., Monkongdee, P., Tasaneeyapan, T., Kanara, N., Kimerling, M.E., Chheng, P., Thai, S., Sar, B., Phanuphak, P., Teeratakulpisarn, N., Phanuphak, N., Dung, N.H., Quy, H.T., Thai, L.H., Varma, J.K., 2010. An Algorithm for Tuberculosis Screening and Diagnosis in People with HIV. N. Engl. J. Med. 362, 707–716. doi:10.1056/NEJMoa0907488

Carpenter, S.M., Nunes-Alves, C., Booty, M.G., Way, S.S., Behar, S.M., 2016. A Higher Activation Threshold of Memory CD8+ T Cells Has a Fitness Cost That Is Modified by TCR Affinity during Tuberculosis. PLoS Pathog. 12. doi:10.1371/journal.ppat.1005380

Catanzano, T.M., 2016. Primary Tuberculosis Imaging: Overview, Radiography, Computed Tomography.

Caws, M., Thwaites, G., Dunstan, S., Hawn, T.R., Lan, N.T.N., Thuong, N.T.T., Stepniewska, K., Huyen, M.N.T., Bang, N.D., Loc, T.H., Gagneux, S., Soolingen, D. van, Kremer, K., Sande, M. van der, Small, P., Anh, P.T.H., Chinh, N.T., Quy, H.T., Duyen, N.T.H., Tho, D.Q., Hieu, N.T., Torok, E., Hien, T.T., Dung, N.H., Nhu,

N.T.Q., Duy, P.M., Chau, N. van V., Farrar, J., 2008. The Influence of Host and Bacterial Genotype on the Development of Disseminated Disease with Mycobacterium tuberculosis. PLOS Pathog 4, e1000034. doi:10.1371/journal.ppat.1000034

Cecconi, F., Levine, B., 2008. The Role of Autophagy in Mammalian Development. Dev. Cell 15, 344–357. doi:10.1016/j.devcel.2008.08.012

Centers for Disease Control and Prevention, 2014. CDC | TB | Fact Sheets - Diagnosis of TB Disease [WWW Document]. URL http://www.cdc.gov/tb/publications/factsheets/testing/diagnosis.htm (accessed 6.6.16).

Chandra, T., Raj, R., Sharma, Y., 2014. Same day sputum smear microscopy approach with modified ZN staining for the diagnosis of pulmonary tuberculosis in a microscopy centre at Rajahmundry. Indian J. Med. Microbiol. 32, 153. doi:10.4103/0255-0857.129801

Chanput, W., Mes, J.J., Wichers, H.J., 2014. THP-1 cell line: An in vitro cell model for immune modulation approach. Int. Immunopharmacol. 23, 37–45. doi:10.1016/j.intimp.2014.08.002

Chen, M., Gan, H., Remold, H.G., 2006. A mechanism of virulence: virulent Mycobacterium tuberculosis strain H37Rv, but not attenuated H37Ra, causes significant mitochondrial inner membrane disruption in macrophages leading to necrosis. J. Immunol. Baltim. Md 1950 176, 3707–3716.

Cliff, J.M., Kaufmann, S.H.E., McShane, H., van Helden, P., O'Garra, A., 2015. The human immune response to tuberculosis and its treatment: a view from the blood. Immunol. Rev. 264, 88–102. doi:10.1111/imr.12269

Cobat, A., Gallant, C.J., Simkin, L., Black, G.F., Stanley, K., Hughes, J., Doherty, T.M., Hanekom, W.A., Eley, B., Jaïs, J.-P., Boland-Auge, A., van Helden, P., Casanova, J.-L., Abel, L., Hoal, E.G., Schurr, E., Alcaïs, A., 2009. Two loci control tuberculin skin test reactivity in an area hyperendemic for tuberculosis. J. Exp. Med. 206, 2583–2591. doi:10.1084/jem.20090892

Coll, R.C., O'Neill, L.A.J., 2010. New insights into the regulation of signalling by toll-like receptors and nod-like receptors. J. Innate Immun. 2, 406–421. doi:10.1159/000315469

Comas, I., Homolka, S., Niemann, S., Gagneux, S., 2009. Genotyping of genetically monomorphic bacteria: DNA sequencing in Mycobacterium tuberculosis highlights the limitations of current methodologies. PloS One 4, e7815. doi:10.1371/journal.pone.0007815

Comstock, G.W., 1978. Tuberculosis in twins: a re-analysis of the Prophit survey. Am. Rev. Respir. Dis. 117, 621–624. doi:10.1164/arrd.1978.117.4.621

Coscolla, M., Gagneux, S., 2014. Consequences of genomic diversity in Mycobacterium tuberculosis. Semin. Immunol. 26, 431–444. doi:10.1016/j.smim.2014.09.012

Crighton, D., Wilkinson, S., O'Prey, J., Syed, N., Smith, P., Harrison, P.R., Gasco, M., Garrone, O., Crook, T., Ryan, K.M., 2006. DRAM, a p53-Induced Modulator of Autophagy, Is Critical for Apoptosis. Cell 126, 121–134. doi:10.1016/j.cell.2006.05.034

Curry, F.J., 2007. Tuberculosis Infection Control Overview, in: Tuberculosis Infection Control - A Practical Manual for Preventing TB. Francis J. Curry National Tuberculosis Center, p. 7.

den Boon, S., van Lill, S.W.P., Borgdorff, M.W., Enarson, D.A., Verver, S., Bateman, E.D., Irusen, E., Lombard, C.J., White, N.W., de Villiers, C., Beyers, N., 2007. High Prevalence of Tuberculosis in Previously Treated Patients, Cape Town, South Africa. Emerg. Infect. Dis. 13, 1189–1194. doi:10.3201/eid1308.051327

Deretic, V., Kimura, T., Timmins, G., Moseley, P., Chauhan, S., Mandell, M., 2015. Immunologic manifestations of autophagy. J. Clin. Invest. 125, 75–84. doi:10.1172/JCI73945

Desikan, P., 2013. Sputum smear microscopy in tuberculosis: Is it still relevant? Indian J. Med. Res. 137, 442–444.

Diel, R., Loddenkemper, R., Nienhaus, A., 2012. Predictive value of interferon-γ release assays and tuberculin skin testing for progression from latent tb infection to disease state: A meta-analysis. Chest 142, 63–75. doi:10.1378/chest.11-3157

Dittrich, N., Berrocal-Almanza, L.C., Thada, S., Goyal, S., Slevogt, H., Sumanlatha, G., Hussain, A., Sur, S., Burkert, S., Oh, D.-Y., Valluri, V., Schumann, R.R., Conrad, M.L., 2015. Toll-like receptor 1 variations influence susceptibility and immune response to Mycobacterium tuberculosis. Tuberculosis 95, 328–335. doi:10.1016/j.tube.2015.02.045

Divangahi, M., Chen, M., Gan, H., Desjardins, D., Hickman, T.T., Lee, D.M., Fortune, S., Behar, S.M., Remold, H.G., 2009. Mycobacterium tuberculosis evades macrophage defenses by inhibiting plasma membrane repair. Nat. Immunol. 10, 899–906. doi:10.1038/ni.1758

Eskelinen, E.-L., 2006. Roles of LAMP-1 and LAMP-2 in lysosome biogenesis and autophagy. Mol. Aspects Med. 27, 495–502. doi:10.1016/j.mam.2006.08.005

Fallow, A., Domenech, P., Reed, M.B., 2010. Strains of the East Asian (W/Beijing) Lineage of Mycobacterium tuberculosis Are DosS/DosT-DosR Two-Component Regulatory System Natural Mutants. J. Bacteriol. 192, 2228–2238. doi:10.1128/JB.01597-09

Ferguson, R.G., 1934. Some Light Thrown on Infection, Resistance and Segregation by a Study of Tuberculosis Among Indians. Trans. Am. Clin. Climatol. Assoc. 50, 18–26.

Fernando, S.L., Saunders, B.M., Sluyter, R., Skarratt, K.K., Goldberg, H., Marks, G.B., Wiley, J.S., Britton, W.J., 2007. A polymorphism in the P2X7 gene increases susceptibility to extrapulmonary tuberculosis. Am. J. Respir. Crit. Care Med. 175, 360–366. doi:10.1164/rccm.200607-970OC

Fernando, S.L., Saunders, B.M., Sluyter, R., Skarratt, K.K., Wiley, J.S., Britton, W.J., 2005. Gene Dosage Determines the Negative Effects of Polymorphic Alleles of the P2X7 Receptor on Adenosine Triphosphate–Mediated Killing of Mycobacteria by Human Macrophages. J. Infect. Dis. 192, 149–155. doi:10.1086/430622

Ferrara, G., Losi, M., D'Amico, R., Roversi, P., Piro, R., Meacci, M., Meccugni, B., Dori, I.M., Andreani, A., Bergamini, B.M., Mussini, C., Rumpianesi, F., Fabbri, L.M., Richeldi, L., 2006. Use in routine clinical practice of two commercial blood tests for diagnosis of infection with Mycobacterium tuberculosis: a prospective study. The Lancet 367, 1328–1334. doi:10.1016/S0140-6736(06)68579-6

Filliol, I., Motiwala, A.S., Cavatore, M., Qi, W., Hazbón, M.H., Bobadilla del Valle, M., Fyfe, J., García-García, L., Rastogi, N., Sola, C., Zozio, T., Guerrero, M.I., León, C.I., Crabtree, J., Angiuoli, S., Eisenach, K.D., Durmaz, R., Joloba, M.L., Rendón, A., Sifuentes-Osornio, J., Ponce de León, A., Cave, M.D., Fleischmann, R., Whittam, T.S., Alland, D., 2006. Global phylogeny of Mycobacterium tuberculosis based on single nucleotide polymorphism (SNP) analysis: insights into tuberculosis evolution, phylogenetic accuracy of other DNA fingerprinting systems, and recommendations for a minimal standard SNP set. J. Bacteriol. 188, 759–772. doi:10.1128/JB.188.2.759-772.2006

Flannagan, R.S., Cosío, G., Grinstein, S., 2009. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. Nat. Rev. Microbiol. 7, 355–366. doi:10.1038/nrmicro2128

Flynn, J.L., Chan, J., 2003. Immune evasion by Mycobacterium tuberculosis: living with the enemy. Curr. Opin. Immunol. 15, 450–455.

Flynn, J.L., Chan, J., 2001. Immunology of Tuberculosis. Annu. Rev. Immunol. 19, 93–129. doi:10.1146/annurev.immunol.19.1.93

Fritzen, A.M., Frøsig, C., Jeppesen, J., Jensen, T.E., Lundsgaard, A.-M., Serup, A.K., Schjerling, P., Proud, C.G., Richter, E.A., Kiens, B., 2016. Role of AMPK in regulation of LC3 lipidation as a marker of autophagy in skeletal muscle. Cell. Signal. 28, 663–674. doi:10.1016/j.cellsig.2016.03.005

Frolov, A., Zielinski, S.E., Crowley, J.R., Dudley-Rucker, N., Schaffer, J.E., Ory, D.S., 2003. NPC1 and NPC2 Regulate Cellular Cholesterol Homeostasis through Generation of Low Density Lipoprotein Cholesterol-derived Oxysterols. J. Biol. Chem. 278, 25517–25525. doi:10.1074/jbc.M302588200

Gagneux, S., 2012. Host–pathogen coevolution in human tuberculosis. Phil Trans R Soc B 367, 850–859. doi:10.1098/rstb.2011.0316

Gagneux, S., Deriemer, K., Van, T., Kato-Maeda, M., de Jong, B.C., Narayanan, S., Nicol, M., Niemann, S., Kremer, K., Gutierrez, M.C., Hilty, M., Hopewell, P.C., Small, P.M., 2006. Variable host-pathogen compatibility in Mycobacterium tuberculosis. Proc.Natl.Acad.Sci.U.S.A 103, 2869–2873.

Gengenbacher, M., Kaufmann, S.H.E., 2012. Mycobacterium tuberculosis: Success through dormancy. Fems Microbiol. Rev. 36, 514–532. doi:10.1111/j.1574-6976.2012.00331.x

Gercken, J., Pryjma, J., Ernst, M., Flad, H.D., 1994. Defective antigen presentation by Mycobacterium tuberculosis-infected monocytes. Infect. Immun. 62, 3472–3478.

Goldberg, M.F., Saini, N.K., Porcelli, S.A., 2014. Evasion of Innate and Adaptive Immunity by Mycobacterium tuberculosis. Microbiol. Spectr. 2. doi:10.1128/microbiolspec.MGM2-0005-2013

Gonzalez, J., Harris, T., Childs, G., Prystowsky, M.B., 2001. Rapamycin blocks IL-2-driven T cell cycle progression while preserving T cell survival. Blood Cells. Mol. Dis. 27, 572–585. doi:10.1006/bcmd.2001.0420

Guirado, E., Schlesinger, L.S., 2013. Modeling the Mycobacterium tuberculosis Granuloma – the Critical Battlefield in Host Immunity and Disease. Front. Immunol. 4. doi:10.3389/fimmu.2013.00098

Gutacker, M.M., Mathema, B., Soini, H., Shashkina, E., Kreiswirth, B.N., Graviss, E.A., Musser, J.M., 2006. Single-Nucleotide Polymorphism–Based Population Genetic Analysis of Mycobacterium tuberculosis Strains from 4 Geographic Sites. J. Infect. Dis. 193, 121–128. doi:10.1086/498574

Gutierrez, M.G., Master, S.S., Singh, S.B., Taylor, G.A., Colombo, M.I., Deretic, V., 2004. Autophagy Is a Defense Mechanism Inhibiting BCG and Mycobacterium tuberculosis Survival in Infected Macrophages. Cell 119, 753–766. doi:10.1016/j.cell.2004.11.038

Hać, A., Domachowska, A., Narajczyk, M., Cyske, K., Pawlik, A., Herman-Antosiewicz, A., 2015. S6K1 controls autophagosome maturation in autophagy induced by sulforaphane or serum deprivation. Eur. J. Cell Biol. 94, 470–481. doi:10.1016/j.ejcb.2015.05.001

Hanekom, M., Gey van Pittius, N.C., McEvoy, C., Victor, T.C., Van Helden, P.D., Warren, R.M., 2011. Mycobacterium tuberculosis Beijing genotype: a template for success. Tuberc. Edinb. Scotl. 91, 510–523. doi:10.1016/j.tube.2011.07.005

Hanekom, M., van der Spuy, G.D., van Pittius, N.C.G., McEvoy, C.R.E., Ndabambi, S.L., Victor, T.C., Hoal, E.G., van Helden, P.D., Warren, R.M., 2007. Evidence that the

Spread of Mycobacterium tuberculosis Strains with the Beijing Genotype Is Human Population Dependent. J. Clin. Microbiol. 45, 2263–2266. doi:10.1128/JCM.02354-06

Hanekom, W.A., Mendillo, M., Manca, C., Haslett, P.A.J., Siddiqui, M.R., Barry, C., Kaplan, G., 2003. Mycobacterium tuberculosis inhibits maturation of human monocyte-derived dendritic cells in vitro. J. Infect. Dis. 188, 257–266. doi:10.1086/376451

Hava, D.L., van der Wel, N., Cohen, N., Dascher, C.C., Houben, D., León, L., Agarwal, S., Sugita, M., van Zon, M., Kent, S.C., Shams, H., Peters, P.J., Brenner, M.B., 2008. Evasion of peptide, but not lipid antigen presentation, through pathogen-induced dendritic cell maturation. Proc. Natl. Acad. Sci. U. S. A. 105, 11281–11286. doi:10.1073/pnas.0804681105

Henderson, R.A., Watkins, S.C., Flynn, J.L., 1997. Activation of human dendritic cells following infection with Mycobacterium tuberculosis. J. Immunol. Baltim. Md 1950 159, 635–643.

Hermans, S., Horsburgh Jr., C.R., Wood, R., 2015. A Century of Tuberculosis Epidemiology in the Northern and Southern Hemisphere: The Differential Impact of Control Interventions. PLoS ONE 10, e0135179. doi:10.1371/journal.pone.0135179

Hershberg, R., Lipatov, M., Small, P.M., Sheffer, H., Niemann, S., Homolka, S., Roach, J.C., Kremer, K., Petrov, D.A., Feldman, M.W., Gagneux, S., 2008. High functional diversity in Mycobacterium tuberculosis driven by genetic drift and human demography. PLoS Biol. 6, e311. doi:10.1371/journal.pbio.0060311

Hingley-Wilson, S.M., Sambandamurthy, V.K., Jacobs, W.R., 2003. Survival perspectives from the world's most successful pathogen, Mycobacterium tuberculosis. Nat. Immunol. 4, 949–955. doi:10.1038/ni981

Hirsh, A.E., Tsolaki, A.G., DeRiemer, K., Feldman, M.W., Small, P.M., 2004. Stable association between strains of Mycobacterium tuberculosis and their human host populations. Proc. Natl. Acad. Sci. U. S. A. 101, 4871–4876. doi:10.1073/pnas.0305627101

Home - Gene - NCBI [WWW Document], n.d. URL http://www.ncbi.nlm.nih.gov/gene/ (accessed 8.2.16).

Houben, E.N., Nguyen, L., Pieters, J., 2006. Interaction of pathogenic mycobacteria with the host immune system. Curr.Opin.Microbiol. 9, 76–85.

Humphreys, I.R., Stewart, G.R., Turner, D.J., Patel, J., Karamanou, D., Snelgrove, R.J., Young, D.B., 2006. A role for dendritic cells in the dissemination of mycobacterial infection. Microbes Infect. Inst. Pasteur 8, 1339–1346. doi:10.1016/j.micinf.2005.12.023

Hur, Y.-G., Gorak-Stolinska, P., Ben-Smith, A., Lalor, M.K., Chaguluka, S., Dacombe, R., Doherty, T.M., Ottenhoff, T.H., Dockrell, H.M., Crampin, A.C., 2013. Combination of Cytokine Responses Indicative of Latent TB and Active TB in Malawian Adults. PLoS ONE 8, e79742. doi:10.1371/journal.pone.0079742

Institute of Medicine (US) Forum on Drug Discovery, D., Science, R.A. of M., 2011. Transmission and Infection Control of Drug-Resistant TB. National Academies Press (US).

Intemann, C.D., Thye, T., Niemann, S., Browne, E.N.L., Chinbuah, M.A., Enimil, A., Gyapong, J., Osei, I., Owusu-Dabo, E., Helm, S., Rüsch-Gerdes, S., Horstmann, R.D., Meyer, C.G., 2009. Autophagy Gene Variant IRGM −261T Contributes to Protection from Tuberculosis Caused by Mycobacterium tuberculosis but Not by M. africanum Strains. PLOS Pathog 5, e1000577. doi:10.1371/journal.ppat.1000577

Ishikawa, E., Ishikawa, T., Morita, Y.S., Toyonaga, K., Yamada, H., Takeuchi, O., Kinoshita, T., Akira, S., Yoshikai, Y., Yamasaki, S., 2009. Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle. J. Exp. Med. 206, 2879–2888. doi:10.1084/jem.20091750

Jepson, A., Fowler, A., Banya, W., Singh, M., Bennett, S., Whittle, H., Hill, A.V.S., 2001. Genetic Regulation of Acquired Immune Responses to Antigens ofMycobacterium tuberculosis: a Study of Twins in West Africa. Infect. Immun. 69, 3989–3994. doi:10.1128/IAI.69.6.3989-3994.2001

Jezbera, J., Hornák, K., Simek, K., 2005. Food selection by bacterivorous protists: insight from the analysis of the food vacuole content by means of fluorescence in situ

hybridization. FEMS Microbiol. Ecol. 52, 351–363. doi:10.1016/j.femsec.2004.12.001

Juárez, E., Carranza, C., Hernández-Sánchez, F., León-Contreras, J.C., Hernández-Pando, R., Escobedo, D., Torres, M., Sada, E., 2012. NOD2 enhances the innate response of alveolar macrophages to Mycobacterium tuberculosis in humans. Eur. J. Immunol. 42, 880–889. doi:10.1002/eji.201142105

Kaufmann, S.H.E., 2002. Protection against tuberculosis: cytokines, T cells, and macrophages. Ann. Rheum. Dis. 61, ii54-ii58. doi:10.1136/ard.61.suppl_2.ii54

Kawamata, T., Kamada, Y., Kabeya, Y., Sekito, T., Ohsumi, Y., 2008. Organization of the Pre-autophagosomal Structure Responsible for Autophagosome Formation. Mol. Biol. Cell 19, 2039–2050. doi:10.1091/mbc.E07-10-1048

Kaye, P.M., Sims, M., Feldmann, M., 1986. Regulation of macrophage accessory cell activity by mycobacteria. II. In vitro inhibition of Ia expression by Mycobacterium microti. Clin. Exp. Immunol. 64, 28–34.

Keane, J., Balcewicz-Sablinska, M.K., Remold, H.G., Chupp, G.L., Meek, B.B., Fenton, M.J., Kornfeld, H., 1997. Infection by Mycobacterium tuberculosis promotes human alveolar macrophage apoptosis. Infect. Immun. 65, 298–304.

Khan, N., Vidyarthi, A., Javed, S., Agrewala, J.N., 2016. Innate Immunity Holding the Flanks until Reinforced by Adaptive Immunity against Mycobacterium tuberculosis Infection. Front. Microbiol. 7. doi:10.3389/fmicb.2016.00328

Kim, J.-J., Lee, H.-M., Shin, D.-M., Kim, W., Yuk, J.-M., Jin, H.S., Lee, S.-H., Cha, G.-H., Kim, J.-M., Lee, Z.-W., Shin, S.J., Yoo, H., Park, Y.K., Park, J.B., Chung, J., Yoshimori, T., Jo, E.-K., 2012. Host cell autophagy activated by antibiotics is required for their effective antimycobacterial drug action. Cell Host Microbe 11, 457–468. doi:10.1016/j.chom.2012.03.008

Kim, Y.E., Hipp, M.S., Bracher, A., Hayer-Hartl, M., Hartl, F.U., 2013. Molecular Chaperone Functions in Protein Folding and Proteostasis. Annu. Rev. Biochem. 82, 323–355. doi:10.1146/annurev-biochem-060208-092442

Kimmey, J.M., Huynh, J.P., Weiss, L.A., Park, S., Kambal, A., Debnath, J., Virgin, H.W., Stallings, C.L., 2015. Unique role for ATG5 in neutrophil-mediated

immunopathology during M. tuberculosis infection. Nature 528, 565–569. doi:10.1038/nature16451

Klopper, M., Warren, R.M., Hayes, C., Gey van Pittius, N.C., Streicher, E.M., Müller, B., Sirgel, F.A., Chabula-Nxiweni, M., Hoosain, E., Coetzee, G., David van Helden, P., Victor, T.C., Trollip, A.P., 2013. Emergence and Spread of Extensively and Totally Drug-Resistant Tuberculosis, South Africa. Emerg. Infect. Dis. 19, 449–455. doi:10.3201/eid1903.120246

Knechel, N.A., 2009. Tuberculosis: Pathophysiology, Clinical Features, and Diagnosis. Crit. Care Nurse 29, 34–43. doi:10.4037/ccn2009968

Koizumi, Y., Toma, C., Higa, N., Nohara, T., Nakasone, N., Suzuki, T., 2012. Inflammasome activation via intracellular NLRs triggered by bacterial infection. Cell. Microbiol. 14, 149–154. doi:10.1111/j.1462-5822.2011.01707.x

Kuballa, P., Nolte, W.M., Castoreno, A.B., Xavier, R.J., 2012. Autophagy and the immune system. Annu. Rev. Immunol. 30, 611–646. doi:10.1146/annurev-immunol-020711-074948

Kumar, D., Nath, L., Kamal, M.A., Varshney, A., Jain, A., Singh, S., Rao, K.V.S., 2010. Genome-wide Analysis of the Host Intracellular Network that Regulates Survival of Mycobacterium tuberculosis. Cell 140, 731–743. doi:10.1016/j.cell.2010.02.012

Kundra, R., Kornfeld, S., 1999. Asparagine-linked oligosaccharides protect Lamp-1 and Lamp-2 from intracellular proteolysis. J. Biol. Chem. 274, 31039–31046.

Laforge, M., Limou, S., Harper, F., Casartelli, N., Rodrigues, V., Silvestre, R., Haloui, H., Zagury, J.-F., Senik, A., Estaquier, J., 2013. DRAM Triggers Lysosomal Membrane Permeabilization and Cell Death in CD4+ T Cells Infected with HIV. PLoS Pathog. 9. doi:10.1371/journal.ppat.1003328

Laird, N.M., Lange, C., 2006. Family-based designs in the age of large-scale gene-association studies. Nat. Rev. Genet. 7, 385–394. doi:10.1038/nrg1839

Lasunskaia, E., Ribeiro, S.C.M., Manicheva, O., Gomes, L.L., Suffys, P.N., Mokrousov, I., Ferrazoli, L., Andrade, M.R.M., Kritski, A., Otten, T., Kipnis, T.L., da Silva, W.D., Vishnevsky, B., Oliveira, M.M., Gomes, H.M., Baptista, I.F., Narvskaya, O., 2010. Emerging multidrug resistant Mycobacterium tuberculosis strains of the Beijing genotype circulating in Russia express a pattern of biological properties associated

with enhanced virulence. Microbes Infect. 12, 467–475. doi:10.1016/j.micinf.2010.02.008

Lee, J., Hartman, M., Kornfeld, H., 2009. Macrophage Apoptosis in Tuberculosis. Yonsei Med. J. 50, 1–11. doi:10.3349/ymj.2009.50.1.1

Lee, S.B., Kim, S., Lee, J., Park, J., Lee, G., Kim, Y., Kim, J.-M., Chung, J., 2007. ATG1, an autophagy regulator, inhibits cell growth by negatively regulating S6 kinase. EMBO Rep. 8, 360–365. doi:10.1038/sj.embor.7400917

Li, C.M., Campbell, S.J., Kumararatne, D.S., Bellamy, R., Ruwende, C., McAdam, K.P.W.J., Hill, A.V.S., Lammas, D.A., 2002. Association of a polymorphism in the P2X7 gene with tuberculosis in a Gambian population. J. Infect. Dis. 186, 1458–1462. doi:10.1086/344351

Li, F., Gao, B., Xu, W., Chen, L., Xiong, S., 2016. The Defect in Autophagy Induction by Clinical Isolates of Mycobacterium Tuberculosis Is Correlated with Poor Tuberculosis Outcomes. PloS One 11, e0147810. doi:10.1371/journal.pone.0147810

Liao, G., Yao, Y., Liu, J., Yu, Z., Cheung, S., Xie, A., Liang, X., Bi, X., 2007. Cholesterol Accumulation Is Associated with Lysosomal Dysfunction and Autophagic Stress in Npc1−/− Mouse Brain. Am. J. Pathol. 171, 962–975. doi:10.2353/ajpath.2007.070052

Look for Gene | Autophagy database [WWW Document], n.d. URL http://www.autophagy.lu/genes/7852 (accessed 12.2.16).

Lory, S., 2014. The Family Mycobacteriaceae, in: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds.), The Prokaryotes. Springer Berlin Heidelberg, pp. 571–575. doi:10.1007/978-3-642-30138-4_339

MacMicking, J.D., Taylor, G.A., McKinney, J.D., 2003. Immune control of tuberculosis by IFN-gamma-inducible LRG-47. Science 302, 654–659. doi:10.1126/science.1088063

MacMicking, J.D., Taylor, G.A., McKinney, J.D., 2003. Immune control of tuberculosis by IFN-gamma-inducible LRG-47. Science 302, 654–659.

Maeß, M.B., Sendelbach, S., Lorkowski, S., 2010. Selection of reliable reference genes during THP-1 monocyte differentiation into macrophages. BMC Mol. Biol. 11, 90. doi:10.1186/1471-2199-11-90

Magee, D.A., Conlon, K.M., Nalpas, N.C., Browne, J.A., Pirson, C., Healy, C., McLoughlin, K.E., Chen, J., Vordermeier, H.M., Gormley, E., MacHugh, D.E., Gordon, S.V., 2014. Innate cytokine profiling of bovine alveolar macrophages reveals commonalities and divergence in the response to Mycobacterium bovis and Mycobacterium tuberculosis infection. Tuberc. Edinb. Scotl. 94, 441–450. doi:10.1016/j.tube.2014.04.004

Mangan, D.F., Wahl, S.M., 1991. Differential regulation of human monocyte programmed cell death (apoptosis) by chemotactic factors and pro-inflammatory cytokines. J. Immunol. Baltim. Md 1950 147, 3408–3412.

Mangan, D.F., Welch, G.R., Wahl, S.M., 1991. Lipopolysaccharide, tumor necrosis factor-alpha, and IL-1 beta prevent programmed cell death (apoptosis) in human peripheral blood monocytes. J. Immunol. Baltim. Md 1950 146, 1541–1546.

Marais, B.J., Mlambo, C.K., Rastogi, N., Zozio, T., Duse, A.G., Victor, T.C., Marais, E., Warren, R.M., 2013. Epidemic Spread of Multidrug-Resistant Tuberculosis in Johannesburg, South Africa. J. Clin. Microbiol. 51, 1818–1825. doi:10.1128/JCM.00200-13

Marais, B.J., Victor, T.C., Hesseling, A.C., Barnard, M., Jordaan, A., Brittle, W., Reuter, H., Beyers, N., van Helden, P.D., Warren, R.M., Schaaf, H.S., 2006. Beijing and Haarlem Genotypes Are Overrepresented among Children with Drug-Resistant Tuberculosis in the Western Cape Province of South Africa. J. Clin. Microbiol. 44, 3539–3543. doi:10.1128/JCM.01291-06

Matsunaga, I., Moody, D.B., 2009. Mincle is a long sought receptor for mycobacterial cord factor. J. Exp. Med. 206, 2865–2868. doi:10.1084/jem.20092533

Mawuenyega, K.G., Forst, C.V., Dobos, K.M., Belisle, J.T., Chen, J., Bradbury, E.M., Bradbury, A.R.M., Chen, X., 2005. Mycobacterium tuberculosis Functional Network Analysis by Global Subcellular Protein Profiling. Mol. Biol. Cell 16, 396–404. doi:10.1091/mbc.E04-04-0329

Mazzaccaro, R.J., Gedde, M., Jensen, E.R., Santen, H.M. van, Ploegh, H.L., Rock, K.L., Bloom, B.R., 1996. Major histocompatibility class I presentation of soluble antigen facilitated by Mycobacterium tuberculosis infection. Proc. Natl. Acad. Sci. 93, 11786–11791.

McDonough, K.A., Kress, Y., Bloom, B.R., 1993a. Pathogenesis of tuberculosis: interaction of Mycobacterium tuberculosis with macrophages. Infect. Immun. 61, 2763–2773.

McDonough, K.A., Kress, Y., Bloom, B.R., 1993b. The interaction of Mycobacterium tuberculosis with macrophages: a study of phagolysosome fusion. Infect. Agents Dis. 2, 232–235.

Meena, L.S., Rajni, 2010. Survival mechanisms of pathogenic Mycobacterium tuberculosis H37Rv. FEBS J. 277, 2416–2427. doi:10.1111/j.1742-4658.2010.07666.x

Meijer, W.H., van der Klei, I.J., Veenhuis, M., Kiel, J.A.K.W., 2007. ATG genes involved in non-selective autophagy are conserved from yeast to man, but the selective Cvt and pexophagy pathways also require organism-specific genes. Autophagy 3, 106–116.

Meyer, C.G., Thye, T., 2014. Host genetic studies in adult pulmonary tuberculosis. Semin. Immunol. 26, 445–453. doi:10.1016/j.smim.2014.09.005

Middelkoop, K., Mathema, B., Myer, L., Shashkina, E., Whitelaw, A., Kaplan, G., Kreiswirth, B., Wood, R., Bekker, L.-G., 2015. Transmission of Tuberculosis in a South African Community With a High Prevalence of HIV Infection. J. Infect. Dis. 211, 53–61. doi:10.1093/infdis/jiu403

Mitchison, D.A., Wallace, J.G., Bhatia, A.L., Selkon, J.B., Subbaiah, T.V., Lancaster, M.C., 1960. A comparison of the virulence in Guinea-pigs of south indian and British tubercle bacilli. Tubercle 41, 1–22. doi:10.1016/S0041-3879(60)80019-0

Möller, M., de Wit, E., Hoal, E.G., 2010. Past, present and future directions in human genetic susceptibility to tuberculosis. FEMS ImmunolMedMicrobiol 58, 3–26.

Morita, D., Miyamoto, A., Hattori, Y., Komori, T., Nakamura, T., Igarashi, T., Harashima, H., Sugita, M., 2013. Th1-skewed tissue responses to a mycolyl glycolipid in

mycobacteria-infected rhesus macaques. Biochem. Biophys. Res. Commun. 441, 108–113. doi:10.1016/j.bbrc.2013.10.021

Mshana, R.N., Hastings, R.C., Krahenbuhl, J.L., 1988. Infection with live mycobacteria inhibits in vitro detection of Ia antigen on macrophages. Immunobiology 177, 40–54. doi:10.1016/S0171-2985(88)80090-1

Munch, Z., Van Lill, S.W.P., Booysen, C.N., Zietsman, H.L., Enarson, D.A., Beyers, N., 2003. Tuberculosis transmission patterns in a high-incidence area: a spatial analysis. Int. J. Tuberc. Lung Dis. 7, 271–277.

Muture, B.N., Keraka, M.N., Kimuu, P.K., Kabiru, E.W., Ombeka, V.O., Oguya, F., 2011. Factors associated with default from treatment among tuberculosis patients in nairobi province, Kenya: A case control study. BMC Public Health 11, 696. doi:10.1186/1471-2458-11-696

National Institute of Allergy and Infectious Disease, 2012. Tuberculosis (TB) Cause [WWW Document]. Tuberculosis. URL https://www.niaid.nih.gov/topics/tuberculosis/understanding/pages/cause.aspx (accessed 6.6.16).

Noss, E.H., Harding, C.V., Boom, W.H., 2000. Mycobacterium tuberculosis inhibits MHC class II antigen processing in murine bone marrow macrophages. Cell. Immunol. 201, 63–74. doi:10.1006/cimm.2000.1633

Odone, A., Riccò, M., Morandi, M., Borrini, B.M., Pasquarella, C., Signorelli, C., 2011. Epidemiology of tuberculosis in a low-incidence Italian region with high immigration rates: differences between not Italy-born and Italy-born TB cases. BMC Public Health 11, 376. doi:10.1186/1471-2458-11-376

Pancholi, P., Mirza, A., Bhardwaj, N., Steinman, R.M., 1993. Sequestration from immune CD4+ T cells of mycobacteria growing in human macrophages. Science 260, 984–986.

Pandey, S., Mittal, R.D., Srivastava, M., Srivastava, K., Singh, S., Srivastava, S., Mittal, B., 2009. Impact of Toll-like receptors [TLR] 2 (-196 to -174 del) and TLR 4 (Asp299Gly, Thr399Ile) in cervical cancer susceptibility in North Indian women. Gynecol. Oncol. 114, 501–505. doi:10.1016/j.ygyno.2009.05.032

Parwati, I., van Crevel, R., van Soolingen, D., 2010. Possible underlying mechanisms for successful emergence of the Mycobacterium tuberculosis Beijing genotype strains. Lancet Infect. Dis. 10, 103–111. doi:10.1016/S1473-3099(09)70330-5

Pasco, P.M., 2012. Diagnostic features of tuberculous meningitis: a cross-sectional study. BMC Res. Notes 5, 49. doi:10.1186/1756-0500-5-49

Patarčić, I., Gelemanović, A., Kirin, M., Kolčić, I., Theodoratou, E., Baillie, K.J., de Jong, M.D., Rudan, I., Campbell, H., Polašek, O., 2015. The role of host genetic factors in respiratory tract infectious diseases: systematic review, meta-analyses and field synopsis. Sci. Rep. 5, 16119. doi:10.1038/srep16119

Paulus, G.L.C., Xavier, R.J., 2015. Autophagy and checkpoints for intracellular pathogen defense: Curr. Opin. Gastroenterol. 31, 14–23. doi:10.1097/MOG.0000000000000134

Petersen, L., Nielsen, G.G., Andersen, P.K., Sørensen, T.I.A., 2002. Case-control study of genetic and environmental influences on premature death of adult adoptees. Genet. Epidemiol. 23, 123–132. doi:10.1002/gepi.1122

Podinovskaia, M., Lee, W., Caldwell, S., Russell, D.G., 2013. Infection of macrophages with Mycobacterium tuberculosis induces global modifications to phagosomal function. Cell. Microbiol. 15, 843–859. doi:10.1111/cmi.12092

Portevin, D., Gagneux, S., Comas, I., Young, D., 2011. Human Macrophage Responses to Clinical Isolates from the Mycobacterium tuberculosis Complex Discriminate between Ancient and Modern Lineages. PLOS Pathog 7, e1001307. doi:10.1371/journal.ppat.1001307

Proikas-Cezanne, T., Takacs, Z., Dönnes, P., Kohlbacher, O., 2015. WIPI proteins: essential PtdIns3P effectors at the nascent autophagosome. J Cell Sci 128, 207–217. doi:10.1242/jcs.146258

Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M.A.R., Bender, D., Maller, J., Sklar, P., de Bakker, P.I.W., Daly, M.J., Sham, P.C., 2007. PLINK: A Tool Set for Whole-Genome Association and Population-Based Linkage Analyses. Am. J. Hum. Genet. 81, 559–575.

Raben, N., Shea, L., Hill, V., Plotz, P., 2009. Monitoring Autophagy in Lysosomal Storage Disorders. Methods Enzymol. 453, 417–449. doi:10.1016/S0076-6879(08)04021-4

Ramos, J.M., Robledano, C., Masiá, M., Belda, S., Padilla, S., Rodríguez, J.C., Gutierrez, F., 2012. Contribution of Interferon gamma release assays testing to the diagnosis of latent tuberculosis infection in HIV-infected patients: A comparison of QuantiFERON-TB Gold In Tube, T-SPOT.TB and tuberculin skin test. BMC Infect. Dis. 12, 169. doi:10.1186/1471-2334-12-169

Ribeiro, S.C.M., Gomes, L.L., Amaral, E.P., Andrade, M.R.M., Almeida, F.M., Rezende, A.L., Lanes, V.R., Carvalho, E.C.Q., Suffys, P.N., Mokrousov, I., Lasunskaia, E.B., 2014. Mycobacterium tuberculosis strains of the modern sublineage of the Beijing family are more likely to display increased virulence than strains of the ancient sublineage. J. Clin. Microbiol. 52, 2615–2624. doi:10.1128/JCM.00498-14

RT2 Profiler PCR Arrays - QIAGEN Online Shop [WWW Document], n.d. URL https://www.qiagen.com/za/shop/pcr/primer-sets/rt2-profiler-pcr-arrays/?catno=PAHS-084Z#geneglobe (accessed 10.4.16).

Russell, D.G., 1995. Mycobacterium and Leishmania: stowaways in the endosomal network. Trends Cell Biol. 5, 125–128.

Sakamoto, K., 2012a. The Pathology of Mycobacterium tuberculosis Infection. Vet. Pathol. Online 49, 423–439. doi:10.1177/0300985811429313

Sakamoto, K., 2012b. The Pathology of Mycobacterium tuberculosis Infection. Vet. Pathol. Online 49, 423–439. doi:10.1177/0300985811429313

Sakowski, E.T., Koster, S., Celhay, C.P., Park, H.S., Shrestha, E., Hetzenecker, S.E., Maurer, K., Cadwell, K., Philips, J.A., 2015. Ubiquilin 1 Promotes IFN-γ-Induced Xenophagy of Mycobacterium tuberculosis. PLOS Pathog 11, e1005076. doi:10.1371/journal.ppat.1005076

Sarkar, R., Lenders, L., Wilkinson, K.A., Wilkinson, R.J., Nicol, M.P., 2012. Modern Lineages of Mycobacterium tuberculosis Exhibit Lineage-Specific Patterns of Growth and Cytokine Induction in Human Monocyte-Derived Macrophages. PLOS ONE 7, e43170. doi:10.1371/journal.pone.0043170

Scott, R.C., Schuldiner, O., Neufeld, T.P., 2004. Role and regulation of starvation-induced autophagy in the Drosophila fat body. Dev. Cell 7, 167–178. doi:10.1016/j.devcel.2004.07.009

Sekati, E.M., Molepo, J., Nchabeleng, M., 2015. Molecular characterisation and associated drug susceptibility patterns of Mycobacterium tuberculosis isolates from South African children. South. Afr. J. Infect. Dis. 30, 11–16.

Seto, S., Tsujimura, K., Koide, Y., 2012. Coronin-1a inhibits autophagosome formation around Mycobacterium tuberculosis-containing phagosomes and assists mycobacterial survival in macrophages. Cell. Microbiol. 14, 710–727. doi:10.1111/j.1462-5822.2012.01754.x

Shin, D.-M., Jeon, B.-Y., Lee, H.-M., Jin, H.S., Yuk, J.-M., Song, C.-H., Lee, S.-H., Lee, Z.-W., Cho, S.-N., Kim, J.-M., Friedman, R.L., Jo, E.-K., 2010a. Mycobacterium tuberculosis Eis Regulates Autophagy, Inflammation, and Cell Death through Redox-dependent Signaling. PLOS Pathog 6, e1001230. doi:10.1371/journal.ppat.1001230

Shin, D.-M., Yuk, J.-M., Lee, H.-M., Lee, S.-H., Son, J.W., Harding, C.V., Kim, J.-M., Modlin, R.L., Jo, E.-K., 2010b. Mycobacterial lipoprotein activates autophagy via TLR2/1/CD14 and a functional vitamin D receptor signalling. Cell. Microbiol. 12, 1648–1665. doi:10.1111/j.1462-5822.2010.01497.x

Shin, J.H., Min, S.-H., Kim, S.-J., Kim, Y.-I., Park, J., Lee, H.K., Yoo, O.J., 2013. TAK1 regulates autophagic cell death by suppressing the phosphorylation of p70 S6 kinase 1. Sci. Rep. 3, 1561. doi:10.1038/srep01561

Shui, W., Petzold, C., Redding, A., Liu, J., Pitcher, A., Sheu, L., Hsieh, T., Keasling, J., Bertozzi, C., 2011. Organelle Membrane Proteomics Reveals Differential Influence of Mycobacterial Lipoglycans on Macrophage Phagosome Maturation and Autophagosome Accumulation. J. Proteome Res. 10, 339–348.

Singh, S.B., Davis, A.S., Taylor, G.A., Deretic, V., 2006. Human IRGM induces autophagy to eliminate intracellular mycobacteria. Science 313, 1438–1441. doi:10.1126/science.1129577

Smith, N.H., Kremer, K., Inwald, J., Dale, J., Driscoll, J.R., Gordon, S.V., van Soolingen, D., Hewinson, R.G., Smith, J.M., 2006. Ecotypes of the Mycobacterium tuberculosis complex. J. Theor. Biol. 239, 220–225. doi:10.1016/j.jtbi.2005.08.036

Song, J.H., Kim, S.Y., Chung, K.S., Moon, C.M., Kim, S.W., Kim, E.Y., Jung, J.Y., Park, M.S., Kim, Y.S., Kim, S.K., Chang, J., Shin, D.J., Kang, Y.A., 2014. Association between genetic variants in the IRGM gene and tuberculosis in a Korean population. Infection 42, 655–660. doi:10.1007/s15010-014-0604-6

Songane, M., Kleinnijenhuis, J., Alisjahbana, B., Sahiratmadja, E., Parwati, I., Oosting, M., Plantinga, T.S., Joosten, L.A.B., Netea, M.G., Ottenhoff, T.H.M., van de Vosse, E., van Crevel, R., 2012. Polymorphisms in autophagy genes and susceptibility to tuberculosis. PloS One 7, e41618. doi:10.1371/journal.pone.0041618

Sørensen, T.I.A., Nielsen, G.G., Andersen, P.K., Teasdale, T.W., 1988. Genetic and Environmental Influences on Premature Death in Adult Adoptees. N. Engl. J. Med. 318, 727–732. doi:10.1056/NEJM198803243181202

Spicer, K.B., Turner, J., Wang, S.-H., Koranyi, K., Powell, D.A., 2015. Tuberculin Skin Testing and T-SPOT.TB in Internationally Adopted Children: Pediatr. Infect. Dis. J. 34, 599–603. doi:10.1097/INF.0000000000000680

Stanley, S.A., Johndrow, J.E., Manzanillo, P., Cox, J.S., 2007. The Type I IFN response to infection with Mycobacterium tuberculosis requires ESX-1-mediated secretion and contributes to pathogenesis. J. Immunol. Baltim. Md 1950 178, 3143–3152.

Strauss, O.J., Warren, R.M., Jordaan, A., Streicher, E.M., Hanekom, M., Falmer, A.A., Albert, H., Trollip, A., Hoosain, E., van Helden, P.D., Victor, T.C., 2008. Spread of a low-fitness drug-resistant Mycobacterium tuberculosis strain in a setting of high human immunodeficiency virus prevalence. J. Clin. Microbiol. 46, 1514–1516. doi:10.1128/JCM.01938-07

Tailleux, L., Neyrolles, O., Honoré-Bouakline, S., Perret, E., Sanchez, F., Abastado, J.-P., Lagrange, P.H., Gluckman, J.C., Rosenzwajg, M., Herrmann, J.-L., 2003. Constrained intracellular survival of Mycobacterium tuberculosis in human dendritic cells. J. Immunol. Baltim. Md 1950 170, 1939–1948.

THE LÜBECK CATASTROPHE, 1931. . Br. Med. J. 1, 986–988.

Theron, G., Zijenah, L., Chanda, D., Clowes, P., Rachow, A., Lesosky, M., Bara, W., Mungofa, S., Pai, M., Hoelscher, M., Dowdy, D., Pym, A., Mwaba, P., Mason, P., Peter, J., Dheda, K., TB-NEAT team, 2014. Feasibility, accuracy, and clinical effect of point-of-care Xpert MTB/RIF testing for tuberculosis in primary-care settings in Africa: a multicentre, randomised, controlled trial. Lancet Lond. Engl. 383, 424–435. doi:10.1016/S0140-6736(13)62073-5

Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T., Tada, K., 1980. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int. J. Cancer 26, 171–176. doi:10.1002/ijc.2910260208

van der Spuy, G.D., Kremer, K., Ndabambi, S.L., Beyers, N., Dunbar, R., Marais, B.J., van Helden, P.D., Warren, R.M., 2009. Changing Mycobacterium tuberculosis population highlights clade-specific pathogenic characteristics. Tuberculosis 89, 120–125. doi:10.1016/j.tube.2008.09.003

van der Wel, N., Hava, D., Houben, D., Fluitsma, D., van Zon, M., Pierson, J., Brenner, M., Peters, P.J., 2007. M. tuberculosis and M. leprae translocate from the phagolysosome to the cytosol in myeloid cells. Cell 129, 1287–1298. doi:10.1016/j.cell.2007.05.059

van Laarhoven, A., Mandemakers, J.J., Kleinnijenhuis, J., Enaimi, M., Lachmandas, E., Joosten, L.A.B., Ottenhoff, T.H.M., Netea, M.G., van Soolingen, D., van Crevel, R., 2013. Low induction of proinflammatory cytokines parallels evolutionary success of modern strains within the Mycobacterium tuberculosis Beijing genotype. Infect. Immun. 81, 3750–3756. doi:10.1128/IAI.00282-13

Vergne, I., Singh, S., Roberts, E., Kyei, G., Master, S., Harris, J., de Haro, S., Naylor, J., Davis, A., Delgado, M., Deretic, V., 2006. Autophagy in immune defense against Mycobacterium tuberculosis. Autophagy 2, 175–178.

Vermeulen, J., De Preter, K., Lefever, S., Nuytens, J., De Vloed, F., Derveaux, S., Hellemans, J., Speleman, F., Vandesompele, J., 2011. Measurable impact of RNA quality on gene expression results from quantitative PCR. Nucleic Acids Res. 39, e63. doi:10.1093/nar/gkr065

Wada, Y., 2013. Vacuoles in mammals. Bioarchitecture 3, 13–19. doi:10.4161/bioa.24126

Wang, H., Sun, H.-Q., Zhu, X., Zhang, L., Albanesi, J., Levine, B., Yin, H., 2015. GABARAPs regulate PI4P-dependent autophagosome:lysosome fusion. Proc. Natl. Acad. Sci. 112, 7015–7020. doi:10.1073/pnas.1507263112

Wang, J.-Y., Hsu, H.-L., Yu, M.-C., Chiang, C.-Y., Yu, F.-L., Yu, C.-J., Lee, L.-N., Yang, P.-C., 2011. Mixed infection with Beijing and non-Beijing strains in pulmonary tuberculosis in Taiwan: prevalence, risk factors, and dominant strain. Clin. Microbiol. Infect. 17, 1239–1245. doi:10.1111/j.1469-0691.2010.03401.x

Wilkinson, R.J., Llewelyn, M., Toossi, Z., Patel, P., Pasvol, G., Lalvani, A., Wright, D., Latif, M., Davidson, R.N., 2000. Influence of vitamin D deficiency and vitamin D receptor polymorphisms on tuberculosis among Gujarati Asians in west London: a case-control study. Lancet Lond. Engl. 355, 618–621. doi:10.1016/S0140-6736(99)02301-6

Wit, E. de, Delport, W., Rugamika, C.E., Meintjes, A., Möller, M., Helden, P.D. van, Seoighe, C., Hoal, E.G., 2010. Genome-wide analysis of the structure of the South African Coloured Population in the Western Cape. Hum. Genet. 128, 145–153. doi:10.1007/s00439-010-0836-1

Wolf, A.J., Linas, B., Trevejo-Nuñez, G.J., Kincaid, E., Tamura, T., Takatsu, K., Ernst, J.D., 2007. Mycobacterium tuberculosis infects dendritic cells with high frequency and impairs their function in vivo. J. Immunol. Baltim. Md 1950 179, 2509–2519.

Wolfe, L.M., Mahaffey, S.B., Kruh, N.A., Dobos, K.M., 2010. Proteomic Definition of the Cell Wall of Mycobacterium tuberculosis. J. Proteome Res. 9, 5816–5826. doi:10.1021/pr1005873

World Health Organization, 2015. Global Tuberculosis Report 2015.

World Health Organization, 2014. Global Tuberculosis Report 2014.

World Health Organization, 2013. Global Tuberculosis Report 2013.

Yorimitsu, T., Zaman, S., Broach, J.R., Klionsky, D.J., 2007. Protein kinase A and Sch9 cooperatively regulate induction of autophagy in Saccharomyces cerevisiae. Mol. Biol. Cell 18, 4180–4189. doi:10.1091/mbc.E07-05-0485

Yuk, J.-M., Shin, D.-M., Lee, H.-M., Yang, C.-S., Jin, H.S., Kim, K.-K., Lee, Z.-W., Lee, S.-H., Kim, J.-M., Jo, E.-K., 2009. Vitamin D3 induces autophagy in human

monocytes/macrophages via cathelicidin. Cell Host Microbe 6, 231–243. doi:10.1016/j.chom.2009.08.004

Zullo, A.J., Lee, S., 2012a. Mycobacterial Induction of Autophagy Varies by Species and Occurs Independently of Mammalian Target of Rapamycin Inhibition. J. Biol. Chem. 287, 12668–12678. doi:10.1074/jbc.M111.320135

Zullo, A.J., Lee, S., 2012b. Mycobacterial induction of autophagy varies by species and occurs independently of mammalian target of rapamycin inhibition. J. Biol. Chem. 287, 12668–12678. doi:10.1074/jbc.M111.320135

APPENDIX I

Table adapted from Möller et al. (2010) is the association studies which investigated TB susceptibility candidate genes.

Gene	Polymorphism	Population	Phenotype	Cases	Controls	p value	Odds Ratio
CCL2	-2518	Ghaha	pTB	2 000	2 300	(1.8×10^{-3})	0.81
		Ghaha	pTB	332 families		(0.01)	0.72
		Russia	pTB	1 400	1 500	Not significant	
		South Africa (SAC)	TB	431	482	Not significant	
	-362	Ghaha	pTB	2 010	2 346	(2.3×10^{-4})	0.83
		Ghaha	pTB	332 families		0.003 (0.004)	0.7
CD209	-871	South Africa (SAC)	pTB	351	360	8.2×10^{-4}	1.85
		Tunisia	TB	138	140	Not significant	
	-336	Colombia	TB	110	299	Not significant	
		Guinea-Bissau	pTB	321	347	Not significant	

Gene	Polymorphism	Population	Phenotype	Cases	Controls	p value	Odds Ratio
		India	pTB	107	157	Not significant	
		South Africa (SAC)	pTB	351	360	0.01	1.48
		Tunisia	TB	138	140	Not significant	
CXC10	-1147	China	TB	240	176	Not significant	
	-872	China	TB	240	176	Not significant	
	-135	China	TB	240	176	0.01	0.51
GC	Gc	Brazil	TB	130	78	Not significant	
		South Africa	TB	281	182	Not significant	
		UK (Gujarati Asian)	TB	123	140	0.009	1.46
IFNG	+874	Pakistan	pTB	111	188	(0.034)	1.46
IL10	-1082	Ghana	pTB	2 010	2 346	Not significant	
		Pakistan	pTB	111	188	Not significant	

Gene	Polymorphism	Population	Phenotype	Cases	Controls	p value	Odds Ratio
	-2849A/-1082A/-819C/-592	Ghana		2 010	129 TST-/2 219 TST+	(0.013)/(0.017)	2.15/2.09
IRF1	5 SNPs	Vietnam	pTB	162	132	Not significant	
	17 SNPs	Indonesia	pTB	192	192	Not significant	
NOS2A	rs9282799 and rs8078340	South Africa (SAC)	TB	431	482	0.011 (0.029)	1.4
	rs2274894	US (African Americans)	TB	279	166	0.003	1.84
		US (Caucasians)	TB	198	123	Not significant	
		US (Caucasians)	TB	198	123	Not significant	
		US (African Americans)	TB	279	166	0.004	1.67

Gene	Polymorphism	Population	Phenotype	Cases	Controls	p value	Odds Ratio
P2RX7	-1513	China (Han Chinese)	TB	96	384	Not significant	
		Gambia	TB	>300	>160	Not significant	
		Liverpool	pTB	86	167	Not significant	
		Mexico	TB	94	100	0.02	5.28
		Russia	pTB	190	128	0.02	1.71
		Sydney	pTB	99	102	Not significant	
	-762	China (Han Chinese)	TB	96	384	Not significant	
		Gambia	TB	>300	>160	0.003	0.70
		Mexico	TB	94	100	Not significant	
		Russia	pTB	190	128	Not significant	
PTPN22	R263Q	Morocco	pTB	123	155	0.01	5.85
	R620W	Morocco	pTB	123	155	0.01	0.14
SLC11A1	rs3731865	US (African Americans)	TB	295	179	0.05	

Gene	Polymorphism	Population	Phenotype	Cases	Controls	p value	Odds Ratio
	rs17221959	US (Caucasians)	TB	237	144	0.04	
	rs3731863	US (Caucasians)	TB	237	144	0.03	
SPI 10	rs2114592	Republic of Guinea	pTB	99 families		0.015	
		Guinea-Bissau	pTB	102 families		Not significant	
		Gambia	pTB	219 families		0.01	
		South Africa (SAC)	TB	381	417	Not significant	
	rs3948464	Russia	pTB	1 912	2 104	Not significant	
		Ghana	pTB	2004	2 366	Not significant	
		Republic of Guinea	pTB	99 families		Not significant	
		Guinea-Bissau	pTB	102 families		Not significant	
		Gambia	pTB	219 families		0.02	

Gene	Polymorphism	Population	Phenotype	Cases	Controls	p value	Odds Ratio
TIRAP	rs8177374	Gambia, Guinea-Bissau, Republic of Guinea	TB	675	605	0.04 (0.013)	0.23
		Colombia	TB	147	391	0.03	0.53
		Russia	pTB	1 867	2 076	Not significant	
		Indonesia	pTB	611	681	Not significant	
		Ghana	pTB	1 913	2 293	Not significant	
		Vietnam	pTB	183	392	Not significant	
		Vietnam	TBM	175	392	0.001	3.02
TLR2	Insertion/deletion (-196 to -174)	Guinea-Bissau	pTB	321	346	0.023	0.70
		US (African Americans)	pTB	295	179	Not significant	
		US (Caucasians)	pTB	237	144	0.0007	0.41

Gene	Polymorphism	Population	Phenotype	Cases	Controls	p value	Odds Ratio
TLR9	rs352143	Guinea-Bissau	pTB	321	346	Not significant	
		US (African Americans)	pTB	295	179	0.029	0.58
		US (Caucasians)	pTB	237	144	0.017	0.53
	rs5743836	Guinea-Bissau	pTB	321	346	Not significant	
		US (African Americans)	pTB	295	179	0.014	0.63
		US (Caucasians)	pTB	237	144	0.05	0.58
TNFRSF1B	rs3397	South Africa (SAC)	TB	429	482	0.049	1.22
	rs3397	Ghana	TB	640	1 158	0.007	1.315
VDR	Fok1	South Africa (SAC)	TB	249	352	Not significant	

Gene	Polymorphism	Population	Phenotype	Cases	Controls	p value	Odds Ratio
		Peru	pTB	103	206	Not significant	
		Meta-analysis	TB	8 studies		inconclusive	
		Meta-analysis (Asia)	TB	12 studies		<0.1	2.0
		Meta-analysis (Africa)	TB	5 studies		Not significant	
		Meta-analysis (South America)	TB	2 studies		Not significant	
	Taq1	South Africa (SAC)	TB	249	352	Not significant	
		Peru	pTB	103	206	Not significant	
		Meta-analysis	TB	8 studies		inconclusive	
		Meta-analysis (Asia)	TB	10 studies		Not significant	
		Meta-analysis (Africa)	TB	8 studies		Not significant	

Gene	Polymorphism	Population	Phenotype	Cases	Controls	p value	Odds Ratio
		Meta-analysis (South America)	TB	2 studies		Not significant	
		South Africa (SAC)	TB	249	352	Not significant	
	Apa1	Meta-analysis (Asia)	TB	6 studies		Not significant	
		Meta-analysis (Africa)	TB	6 studies		Not significant	
	Bsm1	Meta-analysis (Asia)	TB	12 studies		<0.1	0.5
		Meta-analysis (Africa)	TB	4 studies		Not significant	

APPENDIX II

1. BACTERIAL CULTURE

7H9 LIQUID MEDIUM

4.7g 7H9 powder

900mL dH_2O

2mL Glycerol

Autoclace at 121°C for 10 min

Aseptically add 100mL BBL® Middlebrook OADC Enrichment (Becton, Dickinson and Company, USAA.) and 2.5mL 20% Tween-80 to the medium when cooled to 50 °C

MIDDLEBROOK 7H11 AGAR PLATES

19g of 7H11 Agar Base powder

900mL dH_2O

5mL Glycerol

Autoclace at 121°C for 10 min

Aseptically add 100mL BBL® Middlebrook OADC Enrichment (Becton, Dickinson and Company, USA) and 2.5mL 20% Tween-80 to the medium when cooled to 50 °C

2. CELL CULTURE

CELL CULTURE MEDIA

To full bottle of RPMI-1640: add 10% FBS and 0.01% L-Glutamine

FREEZING MEDIA

0.8mL DMSO (Dimethyl sulfoxide)

3.2mL RPMI-1640

3. RT-QPCR SAMPLE PREPERATION

550µL RT^2 SYBR® Green ROX™ qPCR Mastermix (Qiagen, Hilden, Germany)

102µL cDNA synthesis reaction (consisting of 11µL cDNA and 91µL RNase-free water)

448µL RNase-free water

1100µL total volume per sample

APPENDIX III

Table AIII.1 Up- and down-regulated genes (≥ 2-fold), in THP-1 cell infected with a LAM 1 (F13) *M.tb* strain compared to uninfected cells.

Genes Over-Expressed		
Position	**Gene Symbol**	**Fold Regulation**
C04	*CDKN2A*	2,5914
G04	*TGM2*	2,8025
G06	*TNF*	27,552
Genes Under-Expressed		
Position	**Gene Symbol**	**Fold Regulation**
A01	*AKT1*	-12,3477
A02	*AMBRA1*	-5,8616
A03	*APP*	-10,8771
A06	*ATG16L1*	-6,9366
A07	*ATG16L2*	-10,1912
A09	*ATG4A*	-2,973
A10	*ATG4B*	-6,029
A11	*ATG4C*	-14,1455
A12	*ATG4D*	-5,8409
B01	*ATG5*	-3,4889
B02	*ATG7*	-4,6985
B03	*ATG9A*	-15,4538
B04	*ATG9B*	-3,6476
B05	*BAD*	-10,1518
B06	*BAK1*	-9,544
B07	*BAX*	-8,7143
B08	*BCL2*	-8,8154
B09	*BCL2L1*	-12,2687
B10	*BECN1*	-10,7617
B12	*BNIP3*	-2,5756
C01	*CASP3*	-2,6964
C02	*CASP8*	-10,1126
C03	*CDKN1B*	-69,9138
C05	*CLN3*	-8,8447

Position	Gene Symbol	Fold Regulation
C06	*CTSB*	-2,5221
C07	*CTSD*	-10,3128
C08	*CTSS*	-2,2966
C10	*DAPK1*	-4,69
C12	*DRAM2*	-3,2826
D01	*EIF2AK3*	-3,5737
D02	*EIF4G1*	-8,7143
D03	*ESR1*	-5,088
D04	*FADD*	-15,9891
D05	*FAS*	-7,1468
D06	*GAA*	-25,4487
D07	*GABARAP*	-5,53
D08	*GABARAPL1*	-3,941
D09	*GABARAPL2*	-2,3232
D10	*HDAC1*	-11,8225
D11	*HDAC6*	-6,8358
D12	*HGS*	-11,4644
E01	*HSP90AA1*	-5,2576
E02	*HSPA8*	-10,2183
E03	*HTT*	-14,9975
E05	*IGF1*	-7,1826
E07	*IRGM*	-2,2445
E08	*LAMP1*	-41,6086
E09	*MAP1LC3A*	-4,4402
E11	*MAPK14*	-23,5738
E12	*MAPK8*	-2,0771
F01	*MTOR*	-2,6162
F03	*NPC1*	-5,1191
F04	*PIK3C3*	-4,0263
F05	*PIK3CG*	-10,0581
F06	*PIK3R4*	-10,6167
F07	*PRKAA1*	-2,0705
F09	*RAB24*	-8,6781
F10	*RB1*	-3,1109
F11	*RGS19*	-28,7893
F12	*RPS6KB1*	-2,376

Position	Gene Symbol	Fold Regulation
G02	*SQSTM1*	-2,1025
G03	*TGFB1*	-12,3058
G05	*TMEM74*	-3,6492
G07	*TNFSF10*	-8,9569
G08	*TP53*	-9,9802
G09	*ULK1*	-10,3937
G10	*ULK2*	-4,686
G11	*UVRAG*	-8,4952
H01	*ACTB*	-5,4257
H02	*B2M*	-2,9957

Table AIII.2 Up- and down-regulated genes (≥ 2-fold), in THP-1 cell infected with a Typical Beijing strain *M.tb* strain compared to uninfected cells.

Genes Over-Expressed		
Position	**Gene Symbol**	**Fold Regulation**
B01	*ATG5*	5,047
B04	*ATG9B*	2,0351
C04	*CDKN2A*	3,818
C09	*CXCR4*	2,593
D03	*ESR1*	2,5417
E04	*IFNG*	3,0022
E06	*INS*	7,5438
E10	*MAP1LC3B*	2,879
G01	*SNCA*	4,4965
G04	*TGM2*	10,5464
G05	*TMEM74*	4,3468
G06	*TNF*	6,8408
G12	*WIPI1*	4,9423
Genes Under-Expressed		
Position	**Gene Symbol**	**Fold Regulation**
A03	*APP*	-2,0288
B08	*BCL2*	-2,024
C03	*CDKN1B*	-3,3141
D04	*FADD*	-2,0135
D06	*GAA*	-2,5449
E02	*HSPA8*	-2,178
E08	*LAMP1*	-3,0319
F05	*PIK3CG*	-3,4848
F12	*RPS6KB1*	-11,378

Table AIII.3 Up- and down-regulated genes (≥ 2-fold), in THP-1 cell infected with an Atypical Beijing strain *M.tb* strain compared to uninfected cells.

Genes Over-Expressed		
Position	**Gene Symbol**	**Fold Regulation**
A04	*ATG10*	2,5638
C04	*CDKN2A*	2,1695
D03	*ESR1*	3,1096
E04	*IFNG*	6,3349
E06	*INS*	8,2401
G01	*SNCA*	6,8905
G04	*TGM2*	2,1831
G05	*TMEM74*	4,2136
G06	*TNF*	3,8538
Genes Under-Expressed		
Position	**Gene Symbol**	**Fold Regulation**
A01	*AKT1*	-6,7215
A03	*APP*	-10,9263
A05	*ATG12*	-3,5375
A06	*ATG16L1*	-2,2159
A07	*ATG16L2*	-2,9339
A08	*ATG3*	-2,7224
B01	*ATG5*	-5,5147
B02	*ATG7*	-3,0287
B03	*ATG9A*	-5,2729
B05	*BAD*	-2,9923
B06	*BAK1*	-2,6157
B07	*BAX*	-4,8479
B08	*BCL2*	-8,0649
B09	*BCL2L1*	-3,6164
B10	*BECN1*	-3,5824
C01	*CASP3*	-3,6063
C02	*CASP8*	-7,3307
C03	*CDKN1B*	-17,3037
C05	*CLN3*	-3,9407
C07	*CTSD*	-7,1654
C08	*CTSS*	-5,3707
C11	*DRAM1*	-2,1319
C12	*DRAM2*	-3,3947
D02	*EIF4G1*	-3,7429
D04	*FADD*	-14,8795
D06	*GAA*	-10,7345
D07	*GABARAP*	-4,679
D09	*GABARAPL2*	-5,1877
D10	*HDAC1*	-3,2912

Position	Gene Symbol	Fold Regulation
D11	*HDAC6*	-3,2732
D12	*HGS*	-3,7232
E01	*HSP90AA1*	-7,3041
E02	*HSPA8*	-6,9759
E03	*HTT*	-4,36
E07	*IRGM*	-2,1449
E08	*LAMP1*	-6,2182
E11	*MAPK14*	-2,9777
F01	*MTOR*	-2,2169
F02	*NFKB1*	-2,2328
F04	*PIK3C3*	-3,1411
F05	*PIK3CG*	-6,459
F06	*PIK3R4*	-2,7837
F07	*PRKAA1*	-2,5734
F10	*RB1*	-2,5707
F11	*RGS19*	-3,4047
G02	*SQSTM1*	-3,3732
G03	*TGFB1*	-3,4455
G09	*ULK1*	-2, 7035

Table AIII.4 Up- and down-regulated genes (≥ 2-fold), in THP-1 cell infected with H37Rv (laboratory *M.tb* strain) compared to uninfected cells.

Genes Over-Expressed		
Position	**Gene Symbol**	**Fold Regulation**
G04	*TGM2*	5,3889
G06	*TNF*	30,8666
Genes Under-Expressed		
Position	**Gene Symbol**	**Fold Regulation**
A01	*AKT1*	-5,1417
A03	*APP*	-6,0796
A05	*ATG12*	-2,212
A06	*ATG16L1*	-5,7009
A07	*ATG16L2*	-3,9893
A08	*ATG3*	-2,4619
A09	*ATG4A*	-2,6121
A11	*ATG4C*	-5,7836
A12	*ATG4D*	-2,6434
B01	*ATG5*	-3,3706
B03	*ATG9A*	-6,0714
B04	*ATG9B*	-3,2187
B05	*BAD*	-3,4185
B06	*BAK1*	-5,006
B07	*BAX*	-3,3917

Position	Gene Symbol	Fold Regulation
B08	*BCL2*	-4,3198
B09	*BCL2L1*	-3,7043
B10	*BECN1*	-5,0601
B12	*BNIP3*	-2,4196
C02	*CASP8*	-8,3135
C03	*CDKN1B*	-7,5592
C05	*CLN3*	-4,1562
C07	*CTSD*	-4,425
C08	*CTSS*	-3,989
C10	*DAPK1*	-5,7006
C12	*DRAM2*	-4,7839
D02	*EIF4G1*	-2,7527
D04	*FADD*	-8,8841
D06	*GAA*	-10,0064
D07	*GABARAP*	-4,3769
D08	*GABARAPL1*	-2,0728
D09	*GABARAPL2*	-3,223
D10	*HDAC1*	-6,5411
D11	*HDAC6*	-4,3704
D12	*HGS*	-5,0784
E01	*HSP90AA1*	-3,6716
E02	*HSPA8*	-5,4643
E03	*HTT*	-4,2711
E05	*IGF1*	-3,4535
E08	*LAMP1*	-5,8531
E11	*MAPK14*	-4,9926
F04	*PIK3C3*	-3,8815
F05	*PIK3CG*	-9,5218
F06	*PIK3R4*	-4,4213
F07	*PRKAA1*	-2,6491
F08	*PTEN*	-3,517
F09	*RAB24*	-2,631
F10	*RB1*	-5,6998
F11	*RGS19*	-5,6064
F12	*RPS6KB1*	-3,2344
G03	*TGFB1*	-3,7591
G07	*TNFSF10*	-7,2098
G08	*TP53*	-11,6278
G09	*ULK1*	-2,7993
G11	*UVRAG*	-4,1693

Table AIII.5 Up- and down-regulated genes (≥ 2-fold), in THP-1 cell infected with CAS/Kili strain compared to uninfected cells.

Genes Over-Expressed		
Position	**Gene Symbol**	**Fold Regulation**
A04	*ATG10*	2,4337
B11	*BID*	2,1934
C11	*DRAM1*	2,3987
D01	*EIF2AK3*	2,154
E06	*INS*	3,5541
F01	*MTOR*	2,3077
F02	*NFKB1*	4,476
F03	*NPC1*	2,0325
G01	*SNCA*	4,3409
G02	*SQSTM1*	2,8082
G04	*TGM2*	14,5343
G06	*TNF*	184,6735
G10	*ULK2*	2,2294
G12	*WIPI1*	3,6899
Genes Under-Expressed		
Position	**Gene Symbol**	**Fold Regulation**
A01	*AKT1*	-5,1417
A03	*APP*	-6,0796
A05	*ATG12*	-2,212
A06	*ATG16L1*	-5,7009
A07	*ATG16L2*	-3,9893
A08	*ATG3*	-2,4619
A09	*ATG4A*	-2,6121
A11	*ATG4C*	-5,7836
A12	*ATG4D*	-2,6434
B01	*ATG5*	-3,3706
B03	*ATG9A*	-6,0714
B04	*ATG9B*	-3,2187
B05	*BAD*	-3,4185
B06	*BAK1*	-5,006
B07	*BAX*	-3,3917
B08	*BCL2*	-4,3198
B09	*BCL2L1*	-3,7043
B10	*BECN1*	-5,0601
B12	*BNIP3*	-2,4196
C02	*CASP8*	-8,3135
C03	*CDKN1B*	-7,5592
C05	*CLN3*	-4,1562

Position	Gene Symbol	Fold Regulation
C07	*CTSD*	-4,425
C08	*CTSS*	-3,989
C10	*DAPK1*	-5,7006
C12	*DRAM2*	-4,7839
D02	*EIF4G1*	-2,7527
D04	*FADD*	-8,8841
D06	*GAA*	-10,0064
D07	*GABARAP*	-4,3769
D08	*GABARAPL1*	-2,0728
D09	*GABARAPL2*	-3,223
D10	*HDAC1*	-6,5411
D11	*HDAC6*	-4,3704
D12	*HGS*	-5,0784
E01	*HSP90AA1*	-3,6716
E02	*HSPA8*	-5,4643
E03	*HTT*	-4,2711
E05	*IGF1*	-3,4535
E08	*LAMP1*	-5,8531
E11	*MAPK14*	-4,9926
F04	*PIK3C3*	-3,8815
F05	*PIK3CG*	-9,5218
F06	*PIK3R4*	-4,4213
F07	*PRKAA1*	-2,6491
F08	*PTEN*	-3,517
F09	*RAB24*	-2,631
F10	*RB1*	-5,6998
F11	*RGS19*	-5,6064
F12	*RPS6KB1*	-3,2344
G03	*TGFB1*	-3,7591
G07	*TNFSF10*	-7,2098
G08	*TP53*	-11,6278
G09	*ULK1*	-2,7993
G11	*UVRAG*	-4,1693

Table AIII.6. Up- and down-regulated genes (≥ 2-fold), in THP-1 cell infected with a LCC *M.tb* strain compared to uninfected cells.

Genes Over-Expressed		
Position	**Gene Symbol**	**Fold Regulation**
A04	*ATG10*	2,231
C04	*CDKN2A*	98,2074
C10	*DAPK1*	5,8355
D03	*ESR1*	13,8411
D05	*FAS*	5,5969
E03	*HTT*	7,3422

Position	Gene Symbol	Fold Regulation
E04	*IFNG*	90,8793
E06	*INS*	20,4327
E07	*IRGM*	38,1538
F05	*PIK3CG*	2,1939
F08	*PTEN*	6,5413
F09	*RAB24*	66,1299
G04	*TGM2*	2,0434
G05	*TMEM74*	32,4174
G06	*TNF*	2,3163
G10	*ULK2*	12,3595
Genes Under-Expressed		
Position	**Gene Symbol**	**Fold Regulation**
A01	*AKT1*	-2,0994
A03	*APP*	-2,6816
A06	*ATG16L1*	-2,1713
A11	*ATG4C*	-2,3132
B03	*ATG9A*	-2,034
B04	*ATG9B*	-2,1092
B06	*BAK1*	-2,2335
B09	*BCL2L1*	-2,3265
B10	*BECN1*	-2,9428
B11	*BID*	-38,6647
B12	*BNIP3*	-28,7239
C01	*CASP3*	-2,4316
C02	*CASP8*	-9,4146
C03	*CDKN1B*	-3,4975
C05	*CLN3*	-23,0796
C06	*CTSB*	-55,9628
C07	*CTSD*	-918,432
C08	*CTSS*	-59,5264
C11	*DRAM1*	-19,3154
C12	*DRAM2*	-126,806
D02	*EIF4G1*	-9,7046
D04	*FADD*	-2,1877
D07	*GABARAP*	-2,7851
D09	*GABARAPL2*	-23,9817
D10	*HDAC1*	-29,4574
D11	*HDAC6*	-11,9766
D12	*HGS*	-15,4336
E01	*HSP90AA1*	-14,0499
E02	*HSPA8*	-4,921
E08	*LAMP1*	-30,1809
E10	*MAP1LC3B*	-21,5454
E11	*MAPK14*	-93,2988
E12	*MAPK8*	-11,8273
F01	*MTOR*	-6,8409
F07	*PRKAA1*	-11,8566
F11	*RGS19*	-55,9914
F12	*RPS6KB1*	-258,28
G01	*SNCA*	-2,2441

G02	*SQSTM1*	-3021,77
G03	*TGFB1*	-361,959
G12	*WIPI1*	-6,0862

Table AIII.7. Up- and down-regulated genes (≥ 2-fold), in THP-1 cell infected with a Haarlem 3 *M.tb* strain compared to uninfected cells.

Genes Over-Expressed		
Position	**Gene Symbol**	**Fold Regulation**
A01	*AKT1*	2,1072
A02	*AMBRA1*	7,0134
A04	*ATG10*	9,8692
A05	*ATG12*	4,3788
A06	*ATG16L1*	2,0374
A07	*ATG16L2*	3,4508
A08	*ATG3*	4,1283
A09	*ATG4A*	6,5217
A10	*ATG4B*	7,2754
A12	*ATG4D*	4,0152
B01	*ATG5*	4,0762
B02	*ATG7*	7,4065
B03	*ATG9A*	2,1749
B04	*ATG9B*	2,0974
B05	*BAD*	3,0974
B07	*BAX*	2,6044
B08	*BCL2*	2,3137
C04	*CDKN2A*	434,4444
C09	*CXCR4*	4,4163
C10	*DAPK1*	25,8146
D01	*EIF2AK3*	6,3061
D03	*ESR1*	61,2293
D04	*FADD*	2,0221
D05	*FAS*	24,7592
D06	*GAA*	4,1324
D08	*GABARAPL1*	5,0875
E03	*HTT*	32,4801
E04	*IFNG*	402,0267
E05	*IGF1*	4,7304
E06	*INS*	90,3891
E07	*IRGM*	168,7827
E09	*MAP1LC3A*	2,7596
F02	*NFKB1*	2,9289
F03	*NPC1*	3,4416
F04	*PIK3C3*	3,6381

Position	Gene Symbol	Fold Regulation
F05	*PIK3CG*	9,7052
F06	*PIK3R4*	3,7219
F08	*PTEN*	28,937
F09	*RAB24*	292,5419
F10	*RB1*	3,2374
G04	*TGM2*	9,0396
G05	*TMEM74*	143,4062
G06	*TNF*	10,2468
G07	*TNFSF10*	8,0554
G08	*TP53*	4,4139
G09	*ULK1*	5,1401
G10	*ULK2*	54,6753
G11	*UVRAG*	6,2558
Genes Under-Expressed		
Position	**Gene Symbol**	**Fold Regulation**
C05	*CLN3*	-4,6719
C06	*CTSB*	-6,503
C07	*CTSD*	-115,5616
C08	*CTSS*	-12,449
C12	*DRAM2*	-19,6232
D02	*EIF4G1*	-2,0661
D09	*GABARAPL2*	-2,0443
D10	*HDAC1*	-4,1962
D11	*HDAC6*	-2,7881
D12	*HGS*	-7,0392
E01	*HSP90AA1*	-3,1586
E08	*LAMP1*	-4,3191
E10	*MAP1LC3B*	-4,7826
E11	*MAPK14*	-16,5413
E12	*MAPK8*	-2,1031
F07	*PRKAA1*	-2,6327
F10	*RB1*	-3,6306
F11	*RGS19*	-24,7875
F12	*RPS6KB1*	-4,8916
G02	*SQSTM1*	-650,8951
G03	*TGFB1*	-69,8094
G12	*WIPI1*	-39,7955

APPENDIX IV

Case-control association data for SNPs selected from the gene expression data.

Results obtained from Plink with regards to our genotyping data in our cohort study. CHR = Chromosome, SNP = SNP identifier, UNADJ = Unadjusted, asymptotic significance value, BONF = Bonferroni adjusted significance value, L95 = Lower 95% interval, U95 = Upper 95% interval.

CHR	SNP	UNADJ	BONF	OR	L95	U95
11	11:618051	0.003191	1	4.608	1.669	12.72
11	11:1325388	0.004159	1	0.4577	0.2682	0.7811
11	11:1355581	0.005597	1	0.4526	0.2583	0.793
11	11:1988393	0.005772	1	0.5839	0.3985	0.8555
11	11:1372980	0.006542	1	0.4743	0.277	0.812
11	11:1912726	0.01314	1	1.338	1.063	1.683
11	11:1677351	0.01337	1	0.6367	0.4453	0.9105
19	19:41851509	0.01426	1	0.7529	0.5999	0.9447
11	11:365636	0.01714	1	0.7531	0.5965	0.9509
11	11:2006183	0.01741	1	2.81	1.199	6.583
11	11:1945153	0.01919	1	0.3985	0.1845	0.8607
17	17:79663137	0.0213	1	3.126	1.185	8.248
11	11:882406	0.02204	1	0.6146	0.4051	0.9323
9	9:21984661	0.02253	1	1.547	1.063	2.252
5	5:40770220	0.02729	1	1.288	1.029	1.612
11	11:1258803	0.02874	1	1.28	1.026	1.596
11	11:788007	0.0294	1	0.7598	0.5934	0.9729
11	11:1667746	0.02953	1	1.414	1.035	1.932
13	13:113975290	0.03152	1	1.375	1.029	1.837
11	11:1272754	0.03156	1	0.4459	0.2135	0.9311
1	1:150724296	0.03185	1	2.148	1.069	4.318
11	11:1263776	0.03185	1	0.7213	0.5352	0.972
11	11:1767225	0.03298	1	2.585	1.08	6.19
5	5:40759997	0.03341	1	0.4731	0.2374	0.9429
11	11:351629	0.03443	1	1.417	1.026	1.957
11	11:1940543	0.03541	1	0.7714	0.6056	0.9824
11	11:1142621	0.03541	1	0.6852	0.4818	0.9745
11	11:2019174	0.03626	1	0.6267	0.4047	0.9705

CHR	SNP	UNADJ	BONF	OR	L95	U95
11	11:1075920	0.03782	1	1.379	1.018	1.866
11	11:2141603	0.03788	1	0.7821	0.6202	0.9863
11	11:2078654	0.03825	1	2.735	1.056	7.086
11	11:825110	0.03855	1	1.314	1.014	1.702
11	11:1941465	0.03946	1	2.921	1.053	8.102
12	12:12870695	0.03949	1	0.7871	0.6268	0.9885
6	6:36073363	0.044	1	0.5982	0.3629	0.9863
22	22:18247811	0.04637	1	1.405	1.005	1.964
11	11:1093945	0.04845	1	1.342	1.002	1.796
5	5:40771216	0.0499	1	1.258	1	1.584
11	11:918793	0.05135	1	0.595	0.353	1.003
11	11:857345	0.05244	1	0.6791	0.4593	1.004
11	11:1082605	0.0529	1	0.3998	0.158	1.012
11	11:1834306	0.05294	1	1.33	0.9964	1.776
11	11:1392578	0.05306	1	1.331	0.9962	1.779
11	11:443587	0.05344	1	0.6553	0.4268	1.006
11	11:332838	0.05454	1	1.74	0.9893	3.061
11	11:703595	0.05773	1	1.987	0.9777	4.037
11	11:1102906	0.05826	1	0.7766	0.5978	1.009
11	11:617571	0.05857	1	0.4335	0.1823	1.031
5	5:40785811	0.05923	1	0.7961	0.6282	1.009
11	11:840363	0.05949	1	1.256	0.9909	1.593
11	11:1307253	0.06031	1	1.754	0.9759	3.153
1	1:32794447	0.06141	1	1.45	0.9824	2.139
11	11:1722011	0.06327	1	0.4484	0.1923	1.045
17	17:57974476	0.06512	1	0.5023	0.2416	1.044
11	11:793588	0.06525	1	1.38	0.9799	1.943
11	11:320805	0.06606	1	0.5992	0.347	1.035
11	11:325800	0.0672	1	0.5543	0.2947	1.043
22	22:18229187	0.06757	1	1.227	0.9853	1.527
11	11:1381807	0.06875	1	1.265	0.9821	1.629
11	11:1945884	0.06928	1	1.247	0.9827	1.583
11	11:2033785	0.06931	1	1.753	0.9566	3.213
11	11:2024544	0.07045	1	1.231	0.9828	1.541
11	11:413651	0.07079	1	1.939	0.9454	3.975
11	11:1491110	0.07134	1	1.461	0.9676	2.207
11	11:336744	0.07217	1	0.7657	0.5724	1.024
11	11:221584	0.07471	1	1.292	0.9748	1.712
11	11:879497	0.07479	1	1.243	0.9785	1.579
11	11:838672	0.07685	1	0.7726	0.5806	1.028
13	13:48943160	0.08067	1	0.4104	0.1511	1.115
11	11:1546301	0.08124	1	0.502	0.2314	1.089

CHR	SNP	UNADJ	BONF	OR	L95	U95
11	11:680866	0.08228	1	1.295	0.9675	1.733
11	11:1384689	0.08229	1	0.6844	0.4463	1.05
11	11:757290	0.08244	1	1.268	0.9699	1.658
11	11:2172805	0.08344	1	0.4187	0.1562	1.122
11	11:1381790	0.08548	1	1.247	0.9696	1.603
11	11:264391	0.08707	1	1.223	0.9711	1.541
11	11:526478	0.08758	1	1.621	0.9313	2.82
11	11:431757	0.08969	1	2.167	0.8871	5.293
11	11:2130717	0.09136	1	1.354	0.9524	1.924
11	11:2153611	0.09238	1	1.371	0.9494	1.979
11	11:1647110	0.09294	1	0.7709	0.569	1.044
11	11:810882	0.0934	1	0.8084	0.6306	1.036
5	5:179257312	0.09368	1	0.4405	0.1689	1.149
11	11:1900670	0.09478	1	0.522	0.2435	1.119
11	11:1478552	0.09855	1	0.7725	0.5687	1.049
13	13:49052890	0.1	1	0.7533	0.5375	1.056
8	8:11701933	0.1014	1	0.7118	0.4739	1.069
22	22:18245932	0.1044	1	1.227	0.9585	1.571
11	11:1547656	0.1046	1	1.43	0.9284	2.204
11	11:822822	0.1048	1	1.214	0.9604	1.535
11	11:1103876	0.1049	1	1.36	0.9379	1.972
20	20:36792557	0.1057	1	1.598	0.9057	2.818
11	11:1346802	0.1057	1	1.208	0.9608	1.518
11	11:376343	0.1077	1	1.509	0.914	2.49
5	5:40790629	0.109	1	1.209	0.9586	1.524
11	11:268940	0.109	1	0.6856	0.4321	1.088
11	11:658514	0.1093	1	1.254	0.9506	1.653
1	1:150727394	0.1095	1	0.6474	0.38	1.103
11	11:2002587	0.1106	1	1.609	0.8971	2.886
11	11:469583	0.1109	1	0.58	0.2969	1.133
13	13:48909481	0.1117	1	1.473	0.914	2.374
11	11:1975784	0.1125	1	1.246	0.9497	1.635
11	11:1520063	0.1132	1	1.349	0.9314	1.954
11	11:1975710	0.1165	1	1.213	0.9531	1.544
11	11:243268	0.1166	1	0.7998	0.6051	1.057
11	11:1384920	0.1173	1	1.223	0.9506	1.574
11	11:2149864	0.1198	1	0.7954	0.5962	1.061
11	11:558719	0.1211	1	1.664	0.8741	3.167
11	11:1691314	0.122	1	1.243	0.9435	1.638
11	11:1686721	0.122	1	1.751	0.8608	3.562
11	11:870524	0.1236	1	1.269	0.937	1.72
20	20:36784770	0.1236	1	0.8341	0.6622	1.051

CHR	SNP	UNADJ	BONF	OR	L95	U95
11	11:1752283	0.1236	1	0.6312	0.3515	1.134
8	8:11702375	0.124	1	0.726	0.4828	1.092
11	11:427727	0.1251	1	0.692	0.4323	1.108
11	11:1447667	0.1254	1	0.8164	0.6299	1.058
11	11:406473	0.1275	1	1.432	0.9023	2.273
11	11:960114	0.1276	1	1.794	0.8458	3.806
11	11:1886660	0.1279	1	0.5724	0.2791	1.174
11	11:1691563	0.1288	1	0.8395	0.6698	1.052
11	11:1489136	0.1291	1	0.8097	0.6165	1.063
11	11:617214	0.1334	1	1.204	0.9449	1.534
11	11:1805193	0.1349	1	1.645	0.8566	3.16
11	11:1435379	0.1355	1	0.8434	0.6745	1.055
5	5:40796045	0.1357	1	0.7841	0.5696	1.079
11	11:643348	0.1373	1	0.8431	0.6732	1.056
11	11:1570708	0.1377	1	0.7135	0.4569	1.114
11	11:1276395	0.1382	1	0.544	0.2433	1.217
11	11:268927	0.1382	1	0.7025	0.4404	1.12
11	11:619761	0.1406	1	1.406	0.8937	2.211
11	11:1213481	0.1424	1	1.325	0.9098	1.929
11	11:775108	0.1442	1	0.7886	0.5733	1.085
13	13:113964148	0.1446	1	0.7624	0.5296	1.098
17	17:66428002	0.1463	1	0.7756	0.5504	1.093
11	11:244552	0.1468	1	0.8259	0.6377	1.069
8	8:11706513	0.1476	1	0.7661	0.5341	1.099
8	8:11700745	0.1484	1	1.749	0.8195	3.732
11	11:2050558	0.1487	1	0.84	0.6629	1.064
11	11:418652	0.1515	1	1.625	0.837	3.153
11	11:805712	0.1523	1	1.187	0.9387	1.501
11	11:2047956	0.1585	1	0.8464	0.6713	1.067
11	11:479858	0.1608	1	1.87	0.7798	4.483
22	22:18240907	0.1611	1	1.414	0.8709	2.297
11	11:772490	0.1617	1	1.213	0.9257	1.589
11	11:1740189	0.1632	1	0.8324	0.6432	1.077
11	11:443731	0.1639	1	0.8501	0.6763	1.069
11	11:572060	0.1639	1	0.5331	0.2199	1.293
11	11:1261518	0.1659	1	0.4859	0.175	1.349
11	11:881656	0.1687	1	1.183	0.9312	1.503
11	11:248002	0.1698	1	0.7905	0.5651	1.106
11	11:1151320	0.1709	1	0.5949	0.2829	1.251
11	11:236091	0.1711	1	1.27	0.902	1.787
11	11:424291	0.1714	1	1.52	0.8341	2.77
11	11:331326	0.1717	1	1.246	0.909	1.708

CHR	SNP	UNADJ	BONF	OR	L95	U95
11	11:393212	0.174	1	1.436	0.8524	2.419
11	11:1481610	0.1746	1	1.16	0.9362	1.438
5	5:179260009	0.1762	1	0.8474	0.6666	1.077
17	17:66427904	0.1771	1	0.8117	0.5995	1.099
11	11:785007	0.1779	1	2.111	0.7119	6.26
11	11:827955	0.1823	1	0.852	0.6734	1.078
11	11:900929	0.1841	1	1.204	0.9153	1.585
17	17:79658100	0.185	1	1.207	0.9138	1.595
11	11:519240	0.1853	1	1.759	0.7626	4.059
11	11:2064896	0.1857	1	1.183	0.9222	1.519
11	11:2066031	0.1857	1	1.183	0.9222	1.519
14	14:102586213	0.1869	1	0.7895	0.5557	1.121
22	22:18235305	0.1872	1	1.156	0.932	1.434
11	11:892760	0.1879	1	1.188	0.9193	1.535
22	22:18221958	0.1882	1	1.179	0.9225	1.508
11	11:2016908	0.1882	1	0.791	0.5579	1.122
10	10:133784886	0.1883	1	1.921	0.7264	5.078
11	11:1888955	0.1901	1	1.395	0.8479	2.294
11	11:339943	0.1909	1	1.159	0.929	1.447
5	5:179246335	0.1913	1	1.346	0.862	2.102
5	5:40773178	0.1925	1	1.928	0.7184	5.174
11	11:645336	0.1933	1	0.8506	0.6665	1.085
11	11:1678014	0.1942	1	0.8579	0.6806	1.081
11	11:2090456	0.195	1	0.8508	0.6663	1.086
11	11:794491	0.1967	1	0.6239	0.3048	1.277
11	11:2014646	0.1967	1	1.21	0.9058	1.618
11	11:429659	0.2001	1	1.159	0.9246	1.454
8	8:109796788	0.2038	1	0.5503	0.2191	1.383
11	11:619789	0.2045	1	0.8588	0.6788	1.086
11	11:2178634	0.2066	1	0.5541	0.2216	1.385
11	11:1675559	0.2066	1	0.8508	0.662	1.093
20	20:36778610	0.2076	1	0.8039	0.5726	1.129
11	11:1034755	0.2076	1	0.7762	0.5235	1.151
11	11:1667948	0.2078	1	0.7928	0.5523	1.138
11	11:1688902	0.208	1	1.394	0.8313	2.337
11	11:365565	0.208	1	1.213	0.8981	1.638
5	5:179245330	0.2081	1	1.212	0.8983	1.636
11	11:531165	0.211	1	1.609	0.7637	3.39
11	11:1854356	0.2111	1	0.7709	0.5127	1.159
11	11:1328351	0.2113	1	1.182	0.9095	1.535
11	11:1383289	0.2151	1	1.165	0.9149	1.485
11	11:249536	0.2157	1	1.36	0.8357	2.215

CHR	SNP	UNADJ	BONF	OR	L95	U95
11	11:1549147	0.2159	1	0.6829	0.3732	1.25
13	13:113958563	0.2169	1	1.389	0.8246	2.339
11	11:392953	0.2173	1	1.39	0.8238	2.345
11	11:285038	0.218	1	1.334	0.8433	2.112
11	11:1943708	0.2187	1	0.8571	0.6703	1.096
11	11:823809	0.2195	1	0.8279	0.6124	1.119
5	5:40762571	0.2213	1	1.604	0.7523	3.419
11	11:1868169	0.2233	1	0.8552	0.6649	1.1
11	11:314323	0.2234	1	0.6631	0.3423	1.285
11	11:1975791	0.2238	1	1.349	0.8327	2.186
11	11:1559338	0.2247	1	1.254	0.8701	1.808
11	11:268020	0.2279	1	0.8626	0.6784	1.097
11	11:566936	0.2287	1	1.242	0.8726	1.769
17	17:79654639	0.2293	1	1.591	0.7461	3.394
11	11:1229227	0.2298	1	0.8718	0.697	1.091
11	11:2169864	0.2305	1	1.173	0.9039	1.521
11	11:2088968	0.2316	1	0.6627	0.3377	1.3
11	11:2001895	0.2338	1	1.188	0.8947	1.577
11	11:873771	0.2342	1	0.8732	0.6985	1.092
11	11:802437	0.2347	1	0.7744	0.5079	1.181
11	11:1883829	0.2348	1	1.155	0.9109	1.463
11	11:1646413	0.2355	1	0.6988	0.3866	1.263
11	11:1323564	0.2357	1	0.8705	0.6922	1.095
20	20:36763836	0.2365	1	1.306	0.8397	2.03
5	5:179254247	0.2374	1	1.149	0.9127	1.446
11	11:332795	0.2383	1	1.337	0.825	2.167
20	20:36773372	0.2387	1	1.191	0.8905	1.593
11	11:399058	0.2396	1	1.229	0.8717	1.732
11	11:511805	0.2411	1	0.8392	0.6259	1.125
11	11:2054045	0.2427	1	0.8666	0.6815	1.102
22	22:18240851	0.2443	1	1.434	0.7817	2.631
11	11:1032840	0.2447	1	1.547	0.7417	3.227
5	5:176726659	0.2451	1	1.63	0.7151	3.717
17	17:66430923	0.2452	1	1.152	0.9075	1.462
11	11:1080391	0.2455	1	1.431	0.7814	2.622
19	19:41843461	0.2461	1	1.156	0.9051	1.475
11	11:621242	0.2475	1	1.276	0.8443	1.928
11	11:290158	0.248	1	0.7888	0.5275	1.18
11	11:1776740	0.2534	1	0.6027	0.2528	1.437
11	11:1103296	0.2541	1	0.8705	0.6858	1.105
17	17:66433181	0.2547	1	0.8445	0.6314	1.13
11	11:1287443	0.2557	1	1.173	0.8908	1.545

CHR	SNP	UNADJ	BONF	OR	L95	U95
11	11:294119	0.2572	1	0.5657	0.2112	1.515
11	11:874038	0.2578	1	0.7396	0.4387	1.247
11	11:1039614	0.2636	1	0.5853	0.2289	1.497
12	12:12873653	0.264	1	1.143	0.9043	1.444
11	11:1242299	0.2648	1	1.137	0.907	1.426
11	11:230368	0.266	1	1.167	0.8892	1.531
13	13:113973832	0.2666	1	0.7857	0.5134	1.202
11	11:836227	0.2668	1	1.312	0.8126	2.117
11	11:1867936	0.2668	1	0.7743	0.4931	1.216
11	11:1995316	0.2669	1	0.8454	0.6286	1.137
11	11:1872754	0.2671	1	1.48	0.7407	2.957
11	11:392634	0.2682	1	1.375	0.7827	2.414
14	14:102559373	0.271	1	0.5365	0.177	1.626
11	11:1888614	0.2716	1	1.154	0.8939	1.49
11	11:658506	0.2719	1	0.8711	0.681	1.114
11	11:244141	0.274	1	0.8542	0.644	1.133
11	11:244129	0.274	1	0.8542	0.644	1.133
11	11:244108	0.274	1	0.8542	0.644	1.133
11	11:244106	0.274	1	0.8542	0.644	1.133
11	11:383538	0.2743	1	1.455	0.7427	2.851
11	11:2126229	0.2746	1	1.152	0.8936	1.485
11	11:1812367	0.2749	1	0.8745	0.6875	1.112
11	11:2043498	0.2751	1	0.8842	0.7089	1.103
11	11:307036	0.2753	1	1.128	0.9084	1.401
11	11:1309447	0.2758	1	1.295	0.8136	2.061
11	11:366031	0.2761	1	1.418	0.7562	2.661
11	11:656523	0.2764	1	1.297	0.8122	2.07
11	11:1483292	0.2773	1	0.663	0.3158	1.392
8	8:11702542	0.2777	1	1.131	0.9053	1.414
11	11:306878	0.2794	1	1.166	0.8829	1.539
11	11:1721089	0.2806	1	1.134	0.9026	1.424
11	11:270715	0.2822	1	0.8395	0.6103	1.155
20	20:36768064	0.2843	1	1.146	0.8932	1.47
13	13:49045713	0.2856	1	1.551	0.6929	3.473
22	22:18222567	0.2878	1	0.8254	0.5795	1.176
11	11:1044938	0.2902	1	0.8751	0.6834	1.121
11	11:1123965	0.2903	1	1.499	0.7077	3.177
11	11:1857042	0.2908	1	0.8702	0.6723	1.126
11	11:244115	0.2917	1	0.859	0.6476	1.139
11	11:838722	0.292	1	0.8877	0.7112	1.108
11	11:1993975	0.2923	1	0.8732	0.6784	1.124
11	11:1246941	0.2924	1	0.8834	0.7012	1.113

CHR	SNP	UNADJ	BONF	OR	L95	U95
11	11:312929	0.2941	1	1.331	0.7802	2.27
11	11:237087	0.2951	1	0.8591	0.6466	1.142
11	11:1101078	0.2953	1	0.8387	0.6032	1.166
11	11:249408	0.2957	1	0.7698	0.4715	1.257
8	8:11705870	0.2959	1	0.7603	0.4547	1.271
11	11:493130	0.296	1	1.572	0.6731	3.67
11	11:259229	0.2978	1	1.162	0.8761	1.54
11	11:674499	0.2983	1	0.8812	0.6943	1.118
11	11:1272800	0.299	1	0.5939	0.2222	1.587
11	11:1029320	0.299	1	0.6603	0.3017	1.445
11	11:263869	0.3025	1	0.8849	0.7013	1.116
1	1:150737776	0.3026	1	1.123	0.9005	1.401
11	11:1626746	0.3035	1	1.667	0.6297	4.415
11	11:1369851	0.3051	1	1.125	0.8981	1.409
11	11:2031257	0.3053	1	1.156	0.8761	1.525
11	11:1782594	0.3063	1	1.216	0.836	1.769
11	11:780395	0.3077	1	0.8273	0.5747	1.191
13	13:48931979	0.308	1	0.7703	0.4665	1.272
11	11:269129	0.3094	1	0.8598	0.6425	1.151
11	11:218391	0.313	1	0.744	0.4189	1.321
11	11:891094	0.314	1	0.6916	0.3374	1.418
11	11:1860424	0.315	1	0.8081	0.5333	1.225
11	11:2167850	0.3158	1	1.27	0.7959	2.028
11	11:1138100	0.3161	1	1.124	0.8942	1.413
5	5:179245749	0.3164	1	1.135	0.8858	1.455
11	11:1870211	0.3172	1	0.8939	0.7175	1.114
11	11:1242250	0.3173	1	0.879	0.6827	1.132
11	11:1849333	0.3177	1	0.8479	0.6135	1.172
11	11:285920	0.3188	1	0.8886	0.7045	1.121
11	11:1013987	0.3194	1	1.281	0.7869	2.085
11	11:1902793	0.32	1	1.184	0.8486	1.653
11	11:249131	0.3201	1	0.7792	0.4764	1.274
11	11:970320	0.3203	1	1.506	0.6717	3.375
11	11:686714	0.3214	1	0.8749	0.6717	1.139
11	11:283646	0.3215	1	1.402	0.7186	2.737
11	11:1031909	0.3219	1	1.266	0.7938	2.02
11	11:723311	0.3221	1	0.7904	0.4961	1.259
11	11:1663578	0.3237	1	0.627	0.2481	1.585
11	11:1590666	0.3251	1	0.894	0.7151	1.118
11	11:1485629	0.3256	1	1.114	0.8979	1.383
12	12:68552522	0.3259	1	0.8863	0.6965	1.128
11	11:1328912	0.326	1	1.281	0.7815	2.1

CHR	SNP	UNADJ	BONF	OR	L95	U95
11	11:1576079	0.3269	1	0.8923	0.7104	1.121
11	11:802379	0.3269	1	1.119	0.894	1.4
6	6:31543827	0.3275	1	1.284	0.7783	2.12
11	11:1535239	0.3279	1	1.118	0.8945	1.396
11	11:2048508	0.3286	1	1.113	0.8978	1.38
11	11:236649	0.3314	1	1.612	0.6152	4.222
5	5:179240910	0.3322	1	1.126	0.8858	1.432
11	11:298562	0.3329	1	1.246	0.7985	1.943
11	11:1216953	0.3364	1	0.8882	0.6974	1.131
11	11:1678532	0.3368	1	1.151	0.8637	1.534
5	5:40774652	0.3376	1	1.154	0.8615	1.545
11	11:1533973	0.3378	1	1.115	0.8925	1.393
11	11:1093685	0.338	1	0.655	0.2756	1.557
11	11:317030	0.3382	1	1.401	0.7025	2.796
17	17:58024275	0.3388	1	1.118	0.8895	1.405
11	11:827713	0.3399	1	1.166	0.8506	1.598
11	11:2068968	0.3412	1	0.8027	0.5106	1.262
5	5:40791884	0.3442	1	0.8886	0.6957	1.135
11	11:1596299	0.3443	1	0.674	0.2976	1.527
1	1:111663040	0.3449	1	0.8339	0.572	1.216
11	11:698886	0.3455	1	1.365	0.715	2.607
11	11:924159	0.3463	1	0.6807	0.3056	1.516
20	20:36760559	0.3467	1	1.121	0.8834	1.423
11	11:1298304	0.3469	1	0.7155	0.3562	1.437
11	11:804366	0.3495	1	0.7719	0.4488	1.328
11	11:557342	0.35	1	0.8463	0.5965	1.201
11	11:1491096	0.3511	1	1.109	0.8924	1.378
11	11:441106	0.3518	1	0.6228	0.2298	1.688
11	11:223823	0.352	1	1.221	0.8018	1.86
11	11:1871189	0.3522	1	1.163	0.846	1.599
11	11:1736513	0.3534	1	0.6954	0.3229	1.498
11	11:1037474	0.3541	1	0.6062	0.2103	1.747
11	11:300364	0.3552	1	1.118	0.8828	1.415
11	11:253390	0.3555	1	0.8226	0.5435	1.245
11	11:365502	0.356	1	1.154	0.8511	1.565
11	11:587014	0.3569	1	0.8498	0.601	1.201
11	11:256978	0.3586	1	0.8004	0.4975	1.288
11	11:252851	0.3586	1	0.8004	0.4975	1.288
11	11:1652985	0.3602	1	1.169	0.837	1.632
11	11:1911785	0.3625	1	0.8156	0.5259	1.265
11	11:1298430	0.363	1	0.6757	0.2903	1.572
11	11:366710	0.3641	1	0.9015	0.7207	1.128

CHR	SNP	UNADJ	BONF	OR	L95	U95
11	11:712611	0.3647	1	0.8237	0.5416	1.253
11	11:1659810	0.3657	1	0.906	0.7315	1.122
1	1:32786845	0.3661	1	1.137	0.8608	1.502
11	11:1105942	0.3662	1	1.155	0.845	1.579
17	17:79651490	0.3681	1	1.242	0.775	1.989
11	11:1150353	0.3687	1	1.108	0.8859	1.386
11	11:229811	0.3711	1	0.7957	0.4823	1.313
11	11:226084	0.3711	1	0.7957	0.4823	1.313
11	11:1521917	0.3734	1	0.9022	0.7193	1.132
11	11:1110237	0.375	1	0.8974	0.7064	1.14
11	11:1423176	0.3752	1	1.107	0.8843	1.386
6	6:36063141	0.3762	1	0.8364	0.5632	1.242
11	11:927020	0.3767	1	0.9013	0.716	1.135
11	11:612843	0.3772	1	1.118	0.8727	1.433
5	5:40767397	0.3773	1	0.895	0.6995	1.145
11	11:2028103	0.3775	1	1.179	0.8182	1.698
11	11:1735469	0.3775	1	1.281	0.7388	2.222
11	11:1070813	0.38	1	1.141	0.8499	1.532
11	11:470331	0.3815	1	0.9037	0.7203	1.134
11	11:511338	0.3824	1	0.8149	0.5148	1.29
11	11:613208	0.3849	1	1.109	0.8785	1.399
9	9:21973844	0.3854	1	1.362	0.6778	2.738
11	11:800921	0.3856	1	0.7895	0.463	1.346
11	11:1505765	0.3857	1	1.197	0.797	1.799
11	11:766479	0.3866	1	1.106	0.8803	1.39
6	6:36054394	0.3866	1	0.8824	0.6648	1.171
11	11:240754	0.3875	1	0.8021	0.4865	1.323
11	11:1874892	0.3875	1	1.216	0.7805	1.894
11	11:353506	0.3875	1	1.45	0.6242	3.368
6	6:36009539	0.3877	1	1.215	0.781	1.891
11	11:2144972	0.3899	1	1.235	0.763	2.001
11	11:2115305	0.3902	1	1.45	0.6214	3.382
11	11:313572	0.3908	1	1.393	0.6535	2.968
11	11:1515271	0.3918	1	1.102	0.882	1.378
11	11:1866012	0.3975	1	0.8884	0.6755	1.169
20	20:36786987	0.3982	1	0.8992	0.7028	1.151
11	11:1871315	0.4011	1	0.8787	0.6498	1.188
11	11:336138	0.4017	1	1.16	0.8199	1.641
9	9:21973857	0.4033	1	1.455	0.6037	3.508
11	11:266921	0.4034	1	0.908	0.7241	1.139
11	11:266804	0.4034	1	0.908	0.7241	1.139
11	11:265777	0.4034	1	0.908	0.7241	1.139

CHR	SNP	UNADJ	BONF	OR	L95	U95
11	11:1690911	0.4037	1	1.205	0.7774	1.869
11	11:336677	0.4043	1	1.214	0.7694	1.916
5	5:40791672	0.4055	1	1.429	0.616	3.317
11	11:266957	0.4073	1	0.9088	0.7248	1.139
11	11:274181	0.4078	1	0.8148	0.5017	1.323
11	11:1430042	0.4086	1	1.119	0.8566	1.463
6	6:31545391	0.4098	1	0.7679	0.4097	1.439
11	11:493245	0.4131	1	0.6659	0.2515	1.763
11	11:944466	0.4142	1	0.858	0.5941	1.239
11	11:1756291	0.4144	1	0.9079	0.7199	1.145
11	11:1674938	0.4145	1	1.106	0.8684	1.408
11	11:1754400	0.4146	1	1.1	0.8749	1.383
11	11:353630	0.4148	1	1.164	0.8078	1.678
11	11:641991	0.4149	1	0.8043	0.4765	1.358
11	11:733344	0.4161	1	1.098	0.877	1.373
11	11:490035	0.4175	1	1.417	0.6101	3.292
11	11:1001383	0.4181	1	0.6164	0.1911	1.988
11	11:528299	0.4188	1	0.7438	0.363	1.524
11	11:2169110	0.419	1	0.9052	0.711	1.153
17	17:66426766	0.419	1	1.342	0.6574	2.74
11	11:933579	0.4192	1	0.7354	0.3488	1.55
11	11:932710	0.4192	1	0.7354	0.3488	1.55
11	11:1941946	0.4211	1	1.099	0.8734	1.382
6	6:31543605	0.4222	1	1.3	0.685	2.467
11	11:1695421	0.4222	1	0.8822	0.6495	1.198
11	11:502013	0.4254	1	0.9006	0.6963	1.165
11	11:365161	0.4261	1	1.117	0.8506	1.467
11	11:2021581	0.4261	1	0.8515	0.5732	1.265
11	11:273153	0.4277	1	1.094	0.8757	1.368
11	11:554047	0.428	1	1.136	0.8287	1.557
5	5:179245500	0.4294	1	1.199	0.7642	1.882
11	11:358027	0.4296	1	1.236	0.7305	2.092
11	11:565873	0.43	1	1.106	0.8615	1.419
11	11:2023057	0.4306	1	1.121	0.8441	1.488
11	11:218906	0.434	1	0.7318	0.3347	1.6
11	11:540604	0.4344	1	1.195	0.7645	1.868
11	11:222625	0.4349	1	0.8457	0.5552	1.288
11	11:619081	0.4351	1	1.407	0.597	3.315
11	11:642259	0.4376	1	0.752	0.3662	1.544
11	11:1475706	0.4377	1	0.8588	0.5847	1.261
11	11:1651917	0.438	1	1.089	0.8778	1.351
11	11:1827200	0.4394	1	0.8795	0.6351	1.218

CHR	SNP	UNADJ	BONF	OR	L95	U95
11	11:642044	0.4397	1	0.8977	0.6827	1.18
14	14:102596765	0.4399	1	1.242	0.7165	2.153
11	11:1746261	0.4413	1	0.8124	0.4787	1.379
20	20:36764623	0.4419	1	0.7227	0.3159	1.654
11	11:1442226	0.4428	1	1.104	0.8572	1.423
17	17:79668135	0.4429	1	0.8875	0.6542	1.204
11	11:1604324	0.4473	1	0.8304	0.5141	1.341
11	11:1608295	0.4501	1	0.8356	0.5243	1.332
8	8:11711059	0.4511	1	0.9103	0.713	1.162
11	11:966609	0.4515	1	0.7037	0.2819	1.757
14	14:102568367	0.4522	1	0.9004	0.6848	1.184
11	11:290233	0.4542	1	0.916	0.7278	1.153
1	1:150727539	0.4544	1	1.099	0.8576	1.41
13	13:48974742	0.4545	1	0.9132	0.7198	1.159
11	11:1109109	0.4562	1	1.185	0.758	1.853
8	8:11707089	0.4562	1	1.264	0.6828	2.339
11	11:1869410	0.4572	1	0.9198	0.7378	1.147
16	16:75602797	0.4578	1	0.8675	0.5962	1.262
6	6:31544642	0.4594	1	0.8852	0.6409	1.223
10	10:133784881	0.4596	1	1.093	0.8632	1.385
11	11:610277	0.4616	1	0.9019	0.6851	1.187
11	11:589564	0.4618	1	1.09	0.867	1.369
11	11:680647	0.4626	1	0.9109	0.7102	1.168
11	11:1105976	0.4633	1	1.092	0.8627	1.383
11	11:1972205	0.4635	1	0.8699	0.5992	1.263
11	11:1608229	0.4635	1	0.92	0.736	1.15
11	11:828784	0.4642	1	0.8927	0.6589	1.21
11	11:1474210	0.4646	1	0.8777	0.6187	1.245
11	11:1137000	0.4653	1	1.089	0.8659	1.37
6	6:36039543	0.4655	1	0.7853	0.4103	1.503
11	11:1787101	0.4678	1	0.7806	0.3999	1.524
11	11:1890990	0.4708	1	0.9246	0.7471	1.144
20	20:36785127	0.4711	1	1.133	0.8065	1.592
11	11:273808	0.4715	1	1.086	0.8677	1.359
11	11:234349	0.4717	1	0.8317	0.5036	1.374
11	11:1681001	0.472	1	1.158	0.7762	1.728
11	11:762791	0.472	1	0.9123	0.7103	1.172
11	11:312251	0.4721	1	1.169	0.7636	1.79
11	11:359476	0.4722	1	0.8952	0.6621	1.211
11	11:733338	0.4733	1	1.09	0.8608	1.381
17	17:66425317	0.4739	1	0.8791	0.6178	1.251
11	11:2118282	0.4759	1	1.237	0.6898	2.216

CHR	SNP	UNADJ	BONF	OR	L95	U95
11	11:852554	0.4768	1	1.253	0.6732	2.332
17	17:66427696	0.4778	1	0.8995	0.6716	1.205
5	5:179258184	0.4791	1	0.8888	0.6411	1.232
11	11:284039	0.4798	1	1.11	0.8316	1.48
11	11:635240	0.4804	1	1.117	0.8212	1.52
20	20:36793983	0.4817	1	0.8979	0.6652	1.212
11	11:1081112	0.4822	1	0.923	0.7383	1.154
11	11:538940	0.4823	1	0.9111	0.7027	1.181
11	11:1734835	0.4828	1	1.089	0.8583	1.382
11	11:952274	0.4837	1	0.7455	0.3277	1.696
11	11:1961747	0.4845	1	1.184	0.7378	1.899
11	11:769188	0.4847	1	1.099	0.8437	1.431
11	11:565840	0.4851	1	0.8482	0.5343	1.347
11	11:1126979	0.4865	1	1.085	0.8628	1.364
11	11:1912750	0.4866	1	0.8808	0.6159	1.259
11	11:1709656	0.4874	1	1.101	0.8398	1.442
17	17:79656843	0.4875	1	0.8439	0.5226	1.363
17	17:78087041	0.4881	1	1.164	0.7581	1.786
5	5:179263065	0.4883	1	0.7424	0.3197	1.724
11	11:327334	0.4891	1	1.116	0.8182	1.521
16	16:87437787	0.4896	1	0.7298	0.2986	1.784
11	11:1010876	0.4903	1	0.8598	0.5598	1.321
11	11:1405829	0.4914	1	1.112	0.8222	1.503
11	11:1532021	0.4929	1	1.081	0.8657	1.349
17	17:78084941	0.4935	1	1.081	0.8654	1.349
11	11:261209	0.4947	1	0.8584	0.5538	1.33
11	11:1690433	0.4955	1	0.831	0.4879	1.415
11	11:566300	0.4957	1	1.09	0.8506	1.397
11	11:1657308	0.4967	1	0.918	0.7173	1.175
11	11:1974257	0.4968	1	1.371	0.552	3.404
20	20:62714025	0.5014	1	1.124	0.7998	1.579
11	11:1771797	0.5024	1	0.9247	0.7358	1.162
11	11:315299	0.5037	1	0.702	0.2489	1.98
5	5:40785919	0.5038	1	0.7106	0.2609	1.935
11	11:254010	0.5042	1	0.8861	0.6214	1.264
11	11:381147	0.5055	1	0.7273	0.2848	1.857
11	11:345595	0.5063	1	0.9285	0.7461	1.156
11	11:1684146	0.5067	1	0.8218	0.4604	1.467
11	11:2107655	0.508	1	0.9084	0.6835	1.207
11	11:971667	0.5092	1	0.9276	0.7421	1.159
11	11:637014	0.5113	1	0.8944	0.641	1.248
11	11:2070333	0.5115	1	0.9293	0.7466	1.157

CHR	SNP	UNADJ	BONF	OR	L95	U95
11	11:1090343	0.5117	1	1.105	0.8207	1.487
11	11:771034	0.5125	1	1.083	0.8529	1.375
11	11:617520	0.513	1	1.206	0.6883	2.112
11	11:1749805	0.5131	1	0.9263	0.7364	1.165
11	11:1730494	0.5136	1	1.144	0.7642	1.712
11	11:1234492	0.5169	1	1.116	0.8008	1.555
11	11:1889102	0.5202	1	1.09	0.8383	1.417
11	11:1242508	0.5224	1	0.9209	0.7154	1.185
11	11:1857751	0.525	1	1.078	0.8552	1.359
11	11:268219	0.5251	1	0.9308	0.7461	1.161
11	11:1480516	0.5268	1	1.168	0.7225	1.887
11	11:1062990	0.5277	1	1.075	0.8588	1.346
16	16:28504181	0.5318	1	0.9329	0.7503	1.16
11	11:2115390	0.5332	1	1.127	0.7733	1.644
20	20:36784455	0.5336	1	0.8114	0.4202	1.567
11	11:1124327	0.5344	1	0.7328	0.2748	1.954
9	9:21967865	0.5346	1	0.7204	0.2559	2.028
11	11:1475499	0.5348	1	1.094	0.8239	1.453
11	11:2035707	0.5349	1	1.142	0.7512	1.735
11	11:1922004	0.5351	1	0.8256	0.4506	1.513
17	17:66449369	0.5363	1	1.101	0.8124	1.491
11	11:969216	0.5373	1	0.9312	0.7426	1.168
14	14:102552773	0.5389	1	1.154	0.7314	1.82
11	11:1600573	0.5412	1	0.9315	0.742	1.17
11	11:716765	0.5439	1	0.9221	0.7096	1.198
11	11:558165	0.5461	1	1.166	0.7088	1.916
11	11:1017574	0.5466	1	0.7974	0.382	1.665
20	20:36768817	0.5469	1	1.122	0.7714	1.632
11	11:222620	0.5473	1	0.9229	0.7108	1.198
11	11:549051	0.5477	1	0.9333	0.7452	1.169
9	9:21987447	0.5486	1	1.072	0.8546	1.344
6	6:36036909	0.5489	1	0.9192	0.6979	1.211
11	11:1262540	0.5492	1	0.8668	0.543	1.384
22	22:18222263	0.5494	1	1.082	0.8359	1.401
11	11:330630	0.5509	1	1.1	0.8039	1.506
11	11:985547	0.5518	1	0.9312	0.7364	1.178
11	11:301301	0.5533	1	0.8643	0.5336	1.4
11	11:1972143	0.5546	1	0.9179	0.6909	1.219
11	11:1963719	0.5549	1	1.072	0.8514	1.349
13	13:48881196	0.5555	1	0.7836	0.3483	1.763
11	11:1855854	0.5559	1	1.072	0.8498	1.353
11	11:2115722	0.5561	1	0.8584	0.5162	1.427

CHR	SNP	UNADJ	BONF	OR	L95	U95
6	6:31545767	0.5563	1	0.8084	0.3981	1.642
11	11:2171601	0.5572	1	1.083	0.8305	1.411
11	11:955638	0.5578	1	0.9341	0.7436	1.173
11	11:1155777	0.559	1	0.7249	0.2464	2.133
11	11:654147	0.5609	1	1.07	0.8515	1.345
11	11:1068900	0.5627	1	1.07	0.8505	1.347
11	11:1849354	0.5628	1	0.9374	0.753	1.167
5	5:179259736	0.5641	1	1.184	0.6669	2.102
11	11:590201	0.5646	1	0.8346	0.4512	1.544
13	13:113973786	0.5651	1	1.197	0.6487	2.209
10	10:133785993	0.5659	1	1.128	0.7475	1.703
11	11:2087220	0.5664	1	1.07	0.8489	1.349
11	11:2119283	0.5681	1	1.149	0.7136	1.849
11	11:1828941	0.5684	1	1.099	0.7946	1.52
11	11:1891974	0.5704	1	0.8937	0.6061	1.318
11	11:2074764	0.5708	1	1.1	0.7916	1.528
11	11:1364644	0.5708	1	1.076	0.8355	1.385
13	13:48909569	0.5735	1	1.086	0.814	1.45
11	11:1805022	0.5738	1	0.8294	0.4322	1.592
11	11:343776	0.5741	1	1.141	0.7207	1.806
11	11:1109671	0.575	1	1.13	0.7365	1.735
11	11:1110308	0.5756	1	1.125	0.745	1.698
11	11:621634	0.5757	1	0.9344	0.7368	1.185
17	17:78085871	0.5761	1	1.253	0.5686	2.76
16	16:87427870	0.5764	1	0.8145	0.3964	1.673
11	11:2071688	0.5771	1	1.106	0.7768	1.574
11	11:904812	0.5772	1	0.7397	0.2562	2.135
11	11:1444169	0.5781	1	1.088	0.8083	1.465
17	17:78091405	0.5789	1	1.076	0.8313	1.392
11	11:324163	0.5793	1	1.072	0.8379	1.372
11	11:350917	0.5806	1	0.9372	0.7447	1.18
11	11:1867281	0.5819	1	0.8585	0.4987	1.478
11	11:970490	0.5825	1	0.9379	0.7461	1.179
17	17:66426858	0.5831	1	0.8817	0.5623	1.382
11	11:1369465	0.5845	1	0.8826	0.5642	1.381
17	17:66422390	0.5862	1	1.063	0.8537	1.323
11	11:2178267	0.5886	1	1.234	0.5759	2.644
11	11:309127	0.5912	1	0.9417	0.7565	1.172
5	5:179250589	0.5916	1	1.303	0.4957	3.424
20	20:36786479	0.5918	1	1.145	0.6977	1.88
20	20:36772128	0.5923	1	0.8817	0.5563	1.398
11	11:981344	0.5941	1	0.7366	0.2394	2.267

CHR	SNP	UNADJ	BONF	OR	L95	U95
11	11:1850995	0.5946	1	0.9147	0.6587	1.27
11	11:647696	0.5953	1	1.152	0.6827	1.946
22	22:18247625	0.5959	1	0.7789	0.3093	1.961
22	22:18225318	0.5967	1	1.093	0.7854	1.522
11	11:365609	0.5977	1	0.9109	0.6439	1.288
11	11:1042981	0.5986	1	0.7535	0.2627	2.161
5	5:179260478	0.6014	1	0.9104	0.6401	1.295
8	8:11716188	0.6022	1	0.7864	0.3185	1.941
5	5:40766503	0.6023	1	0.8428	0.443	1.603
11	11:1891905	0.6024	1	1.078	0.8123	1.431
11	11:1622153	0.6031	1	1.064	0.8415	1.346
11	11:866920	0.6035	1	1.184	0.6266	2.235
11	11:1911439	0.6041	1	0.9103	0.6382	1.299
17	17:79664426	0.6047	1	0.9398	0.743	1.189
11	11:2024559	0.6076	1	0.8623	0.4899	1.518
11	11:1716153	0.6076	1	0.9416	0.7484	1.185
17	17:79662836	0.6079	1	0.8091	0.3601	1.818
11	11:1082319	0.6084	1	0.8457	0.4453	1.606
11	11:247897	0.6093	1	0.9165	0.6559	1.281
11	11:654043	0.6096	1	0.877	0.5299	1.451
5	5:179258176	0.6102	1	1.094	0.775	1.544
11	11:1837050	0.6103	1	0.9388	0.7363	1.197
5	5:179250130	0.6106	1	1.205	0.5878	2.471
11	11:618172	0.6108	1	1.063	0.8405	1.344
11	11:1801823	0.6112	1	1.101	0.7607	1.592
11	11:2182224	0.6114	1	0.9399	0.7399	1.194
11	11:1941996	0.613	1	1.124	0.7146	1.768
11	11:268183	0.615	1	0.9418	0.7456	1.19
11	11:1263374	0.6157	1	0.8876	0.5573	1.414
11	11:492972	0.6165	1	1.253	0.5184	3.029
11	11:1728138	0.6178	1	1.087	0.7827	1.511
11	11:833872	0.6238	1	1.057	0.8475	1.318
11	11:1320672	0.6251	1	0.8574	0.4626	1.589
6	6:36009833	0.6273	1	0.9477	0.763	1.177
11	11:1092388	0.6273	1	1.102	0.7447	1.631
11	11:2125911	0.6274	1	1.071	0.813	1.41
11	11:2132045	0.6275	1	1.185	0.5964	2.356
11	11:772701	0.6292	1	1.055	0.848	1.313
11	11:999764	0.631	1	0.9012	0.5894	1.378
16	16:87426655	0.6312	1	0.8862	0.5412	1.451
11	11:463577	0.6319	1	1.193	0.5798	2.454
16	16:28488944	0.6322	1	1.19	0.583	2.431

CHR	SNP	UNADJ	BONF	OR	L95	U95
14	14:102589849	0.6327	1	0.8003	0.321	1.995
11	11:609451	0.6332	1	0.8165	0.3551	1.878
6	6:35996568	0.6346	1	1.198	0.5694	2.519
13	13:48972880	0.6396	1	1.204	0.5537	2.618
11	11:493007	0.6404	1	1.063	0.8225	1.374
11	11:1824791	0.6405	1	0.9216	0.6543	1.298
11	11:336192	0.6407	1	1.077	0.7891	1.47
11	11:684056	0.6416	1	1.057	0.8378	1.333
11	11:2048485	0.6421	1	1.259	0.4768	3.324
11	11:1714912	0.6426	1	1.188	0.5737	2.46
9	9:21981583	0.6431	1	0.9209	0.6499	1.305
16	16:28503698	0.647	1	1.187	0.5706	2.468
11	11:391421	0.6471	1	1.202	0.5464	2.645
11	11:1632180	0.648	1	1.053	0.8433	1.315
11	11:346580	0.6483	1	0.8023	0.3114	2.067
11	11:1533155	0.6495	1	1.255	0.4716	3.339
11	11:2110046	0.6506	1	0.9225	0.6507	1.308
9	9:21974218	0.6514	1	1.083	0.7668	1.529
11	11:335885	0.6529	1	1.059	0.8257	1.357
11	11:1629693	0.6536	1	1.082	0.7668	1.527
11	11:2021493	0.6551	1	0.8249	0.3545	1.919
11	11:2095799	0.6566	1	0.9487	0.752	1.197
11	11:1776332	0.6568	1	0.9019	0.5719	1.422
11	11:1676922	0.657	1	1.05	0.8457	1.305
11	11:1804431	0.6576	1	0.9236	0.6499	1.313
11	11:1499084	0.6588	1	1.05	0.8456	1.304
11	11:1681265	0.6589	1	1.182	0.5618	2.489
16	16:28490517	0.6612	1	1.057	0.8254	1.353
11	11:1871245	0.6618	1	0.9265	0.6583	1.304
11	11:1969888	0.6644	1	1.052	0.8375	1.321
11	11:1811496	0.6645	1	0.9165	0.6181	1.359
11	11:1868236	0.6686	1	1.089	0.7382	1.605
11	11:895596	0.6691	1	1.083	0.751	1.562
11	11:396546	0.6697	1	0.9523	0.7607	1.192
11	11:2156536	0.6704	1	0.9532	0.7646	1.188
11	11:1133367	0.6709	1	1.053	0.8285	1.34
22	22:18250895	0.6718	1	1.235	0.4646	3.284
13	13:48992810	0.6726	1	1.061	0.8062	1.396
11	11:996522	0.6738	1	1.061	0.8054	1.398
11	11:1529538	0.6746	1	0.9199	0.6231	1.358
20	20:36780752	0.6748	1	0.9378	0.6947	1.266
6	6:36006998	0.6761	1	0.9467	0.7322	1.224

CHR	SNP	UNADJ	BONF	OR	L95	U95
9	9:21978358	0.677	1	0.8833	0.4927	1.584
11	11:2073913	0.6782	1	1.059	0.8083	1.387
11	11:1062197	0.6785	1	0.9456	0.726	1.232
11	11:2169774	0.6791	1	1.053	0.8232	1.348
11	11:1242227	0.6827	1	1.046	0.8417	1.301
11	11:1961869	0.684	1	1.05	0.8295	1.33
11	11:462105	0.6854	1	0.9307	0.6576	1.317
11	11:733639	0.6869	1	0.9544	0.7604	1.198
11	11:236370	0.6883	1	0.9114	0.5792	1.434
11	11:1280238	0.69	1	0.9275	0.6407	1.343
11	11:366251	0.6901	1	0.9242	0.6272	1.362
11	11:248855	0.6901	1	1.156	0.5675	2.354
11	11:1714061	0.6903	1	1.046	0.8372	1.308
11	11:356827	0.691	1	0.9405	0.6952	1.272
11	11:2116374	0.6921	1	1.199	0.4886	2.942
11	11:1408590	0.6935	1	1.046	0.8363	1.308
11	11:233067	0.6938	1	1.074	0.7523	1.534
11	11:1325829	0.6945	1	1.124	0.6281	2.01
11	11:1558149	0.6957	1	1.13	0.6133	2.08
11	11:1648841	0.6963	1	0.9186	0.5998	1.407
8	8:11703853	0.6968	1	0.8734	0.442	1.726
11	11:718436	0.6972	1	1.149	0.571	2.311
17	17:66422388	0.6991	1	1.051	0.8153	1.356
11	11:1801578	0.7006	1	0.9152	0.5825	1.438
17	17:66422158	0.7022	1	0.9454	0.7088	1.261
11	11:1516110	0.7038	1	1.059	0.789	1.421
13	13:48882831	0.7039	1	0.9468	0.7144	1.255
5	5:179264132	0.7041	1	0.9088	0.5548	1.489
11	11:686310	0.7042	1	1.158	0.5431	2.469
11	11:1125071	0.7046	1	1.127	0.6063	2.096
11	11:884543	0.7054	1	0.9416	0.6892	1.286
11	11:1688679	0.7067	1	0.9412	0.6864	1.291
11	11:299091	0.7082	1	1.044	0.8337	1.307
11	11:2034099	0.7091	1	1.043	0.8348	1.304
17	17:66419633	0.7104	1	0.9312	0.639	1.357
11	11:1628844	0.7115	1	0.9449	0.7	1.276
11	11:1245298	0.7118	1	1.084	0.7077	1.659
11	11:1494225	0.7127	1	1.114	0.6278	1.976
11	11:1005308	0.7138	1	0.9564	0.7535	1.214
11	11:1531866	0.7158	1	0.922	0.5955	1.428
11	11:1060123	0.7164	1	0.9118	0.554	1.501
11	11:1549955	0.7194	1	0.9091	0.5404	1.529

CHR	SNP	UNADJ	BONF	OR	L95	U95
17	17:66436066	0.7199	1	1.052	0.7969	1.389
11	11:287070	0.7209	1	0.9185	0.5762	1.464
11	11:1823725	0.7251	1	1.045	0.8173	1.336
11	11:1637127	0.7252	1	0.8813	0.4355	1.783
20	20:36775107	0.7254	1	0.904	0.5148	1.587
11	11:1739337	0.7256	1	0.8454	0.3309	2.16
22	22:18236880	0.7268	1	1.157	0.5099	2.627
11	11:521302	0.7272	1	0.8248	0.2795	2.434
11	11:2135137	0.7273	1	1.054	0.7833	1.419
11	11:1111474	0.7301	1	1.06	0.7611	1.477
11	11:2169014	0.733	1	0.9514	0.7145	1.267
11	11:1857270	0.7337	1	0.9352	0.6355	1.376
9	9:21990457	0.7351	1	1.046	0.8054	1.359
11	11:662407	0.7352	1	0.9609	0.7624	1.211
11	11:1507731	0.7354	1	0.8883	0.447	1.766
11	11:1471286	0.7359	1	1.04	0.8265	1.31
11	11:1714464	0.7365	1	0.9597	0.755	1.22
11	11:2139284	0.7368	1	1.145	0.5201	2.52
11	11:456234	0.7374	1	0.8848	0.4327	1.809
8	8:11707027	0.7376	1	1.038	0.8351	1.29
11	11:232641	0.7377	1	1.063	0.742	1.524
11	11:1890433	0.7378	1	1.103	0.6223	1.954
11	11:1401890	0.738	1	0.9576	0.7432	1.234
11	11:833668	0.739	1	1.039	0.8282	1.304
11	11:2036011	0.7395	1	0.9123	0.531	1.567
11	11:350275	0.7398	1	1.038	0.8348	1.289
11	11:1859518	0.7418	1	0.9054	0.5011	1.636
11	11:1736664	0.7421	1	0.9555	0.7287	1.253
11	11:1263467	0.7423	1	0.8665	0.3687	2.036
20	20:36793529	0.745	1	0.9593	0.7466	1.232
11	11:1019194	0.7456	1	0.9282	0.5916	1.456
1	1:111660540	0.7463	1	0.9636	0.7696	1.207
11	11:248181	0.7465	1	1.062	0.7379	1.528
11	11:1868554	0.7468	1	0.9553	0.7235	1.261
11	11:370252	0.7491	1	0.8929	0.4459	1.788
11	11:1650156	0.7534	1	0.9627	0.7594	1.22
13	13:48886282	0.7535	1	1.039	0.8188	1.318
11	11:2120123	0.7539	1	0.8714	0.3686	2.06
11	11:420011	0.754	1	1.113	0.5703	2.171
11	11:372157	0.7544	1	0.9634	0.7628	1.217
11	11:1574375	0.7544	1	0.9647	0.7702	1.208
11	11:593042	0.7555	1	1.139	0.5024	2.582

CHR	SNP	UNADJ	BONF	OR	L95	U95
11	11:970503	0.7566	1	0.9598	0.7406	1.244
11	11:1447683	0.757	1	1.097	0.61	1.973
11	11:1641031	0.7578	1	1.036	0.8292	1.293
11	11:324368	0.7585	1	0.9047	0.478	1.713
11	11:356871	0.7611	1	1.039	0.8126	1.328
11	11:624851	0.7611	1	0.869	0.3513	2.149
11	11:1323284	0.7622	1	1.08	0.6566	1.776
17	17:78079669	0.7626	1	1.035	0.8292	1.291
11	11:1628029	0.7632	1	0.8751	0.3672	2.085
19	19:41845872	0.7634	1	1.057	0.7354	1.52
11	11:696612	0.7639	1	0.8802	0.3827	2.024
11	11:2038654	0.765	1	1.039	0.809	1.334
11	11:323649	0.7663	1	1.053	0.7484	1.482
6	6:36063119	0.7679	1	0.9118	0.4938	1.684
11	11:1263523	0.7683	1	0.9287	0.5675	1.52
11	11:827440	0.7692	1	0.8937	0.4218	1.893
22	22:18226764	0.7706	1	1.077	0.6541	1.773
11	11:274799	0.7714	1	0.9618	0.7396	1.251
11	11:295343	0.7721	1	1.035	0.8209	1.305
11	11:1909006	0.773	1	0.9573	0.7116	1.288
11	11:627517	0.778	1	1.11	0.5379	2.289
11	11:1690466	0.7789	1	1.04	0.7916	1.366
11	11:1828987	0.779	1	0.9246	0.5349	1.598
11	11:1124629	0.7798	1	1.111	0.5321	2.318
11	11:1009641	0.7799	1	0.9262	0.5408	1.586
11	11:1760768	0.7803	1	0.9482	0.6526	1.378
11	11:297359	0.7808	1	1.036	0.8071	1.33
11	11:430339	0.7814	1	1.143	0.4458	2.929
11	11:1296237	0.7817	1	0.9312	0.5625	1.542
11	11:305624	0.7823	1	0.9625	0.7341	1.262
11	11:1717577	0.7846	1	0.9658	0.7525	1.24
11	11:1021268	0.7857	1	0.8824	0.358	2.175
8	8:11710174	0.7857	1	1.083	0.6096	1.924
11	11:252649	0.7859	1	0.9652	0.7476	1.246
11	11:1640465	0.7861	1	0.9675	0.7624	1.228
11	11:1870125	0.7874	1	1.075	0.6354	1.819
11	11:1443184	0.788	1	1.046	0.7541	1.451
11	11:816233	0.7886	1	0.9358	0.5762	1.52
11	11:1545198	0.7886	1	0.8989	0.4123	1.959
11	11:2151818	0.7902	1	0.9593	0.7063	1.303
11	11:2111990	0.7909	1	1.129	0.4607	2.766
11	11:1493132	0.7913	1	0.9445	0.6189	1.441

CHR	SNP	UNADJ	BONF	OR	L95	U95
11	11:1088835	0.7914	1	1.043	0.7656	1.42
13	13:48901965	0.7915	1	1.055	0.7107	1.565
13	13:48942143	0.7915	1	1.055	0.7107	1.565
16	16:28488943	0.7916	1	0.9667	0.7523	1.242
17	17:66449122	0.7918	1	0.959	0.703	1.308
11	11:291694	0.7919	1	0.9706	0.7779	1.211
11	11:288115	0.7923	1	1.036	0.7953	1.35
8	8:11711516	0.7926	1	0.8895	0.3716	2.129
11	11:1276327	0.794	1	0.9524	0.6606	1.373
5	5:179249110	0.7947	1	0.9539	0.6682	1.362
11	11:252616	0.7972	1	1.052	0.7152	1.547
11	11:1575068	0.7999	1	1.039	0.7752	1.391
11	11:1233148	0.8017	1	0.9437	0.6006	1.483
11	11:1018156	0.8028	1	1.072	0.6224	1.845
11	11:285936	0.8032	1	1.074	0.6145	1.875
11	11:1093769	0.807	1	1.046	0.7275	1.505
11	11:419794	0.8074	1	0.9368	0.5541	1.584
17	17:78093353	0.8075	1	1.041	0.7556	1.433
11	11:1870852	0.8078	1	1.028	0.8255	1.279
11	11:1660751	0.808	1	0.9466	0.6081	1.473
17	17:66430889	0.8094	1	0.9515	0.6356	1.425
11	11:490811	0.8094	1	0.9693	0.7526	1.248
11	11:1473931	0.8114	1	1.029	0.8161	1.296
6	6:36013607	0.8125	1	0.8938	0.3536	2.26
11	11:559016	0.8149	1	0.971	0.7589	1.242
11	11:1408892	0.8157	1	0.9736	0.7775	1.219
5	5:176740244	0.816	1	1.028	0.8176	1.291
11	11:845776	0.8166	1	0.9599	0.6794	1.356
11	11:231866	0.8186	1	1.043	0.7287	1.492
11	11:1718266	0.8187	1	0.9585	0.6668	1.378
17	17:79655251	0.819	1	0.9729	0.7691	1.231
16	16:28488682	0.8194	1	0.9087	0.3995	2.067
11	11:1238828	0.8229	1	1.025	0.8228	1.278
11	11:1242690	0.8232	1	1.027	0.812	1.299
9	9:21986218	0.8238	1	0.9676	0.724	1.293
11	11:2042162	0.8243	1	0.9452	0.575	1.554
22	22:18250968	0.8251	1	1.025	0.821	1.281
11	11:2115130	0.8259	1	1.093	0.4953	2.411
11	11:1093574	0.8261	1	0.9398	0.54	1.635
11	11:549080	0.8263	1	1.027	0.8066	1.309
11	11:390499	0.8271	1	1.11	0.4353	2.83
11	11:353757	0.8286	1	0.9753	0.7778	1.223

CHR	SNP	UNADJ	BONF	OR	L95	U95
11	11:1765156	0.8314	1	0.9595	0.6559	1.404
11	11:1215795	0.833	1	0.9146	0.399	2.097
13	13:49051012	0.8344	1	0.9662	0.6998	1.334
14	14:102560631	0.8352	1	0.9596	0.6509	1.415
11	11:619390	0.8354	1	0.9105	0.3762	2.204
20	20:36768258	0.8357	1	1.056	0.6295	1.772
11	11:1985127	0.8369	1	0.9735	0.7539	1.257
11	11:300047	0.8373	1	0.9773	0.785	1.217
11	11:2076043	0.8386	1	1.03	0.7778	1.363
11	11:357903	0.8387	1	0.8994	0.3242	2.495
11	11:1522125	0.8389	1	1.052	0.6429	1.723
11	11:967659	0.8399	1	0.9386	0.5077	1.735
11	11:1148459	0.8435	1	1.036	0.7282	1.474
11	11:653968	0.8436	1	1.023	0.8166	1.281
11	11:803401	0.8452	1	0.9542	0.596	1.528
11	11:830497	0.8453	1	1.086	0.4741	2.488
11	11:269856	0.8454	1	1.03	0.7633	1.391
11	11:1230393	0.8457	1	0.9687	0.7034	1.334
17	17:66429466	0.8458	1	1.036	0.7237	1.484
11	11:702250	0.8463	1	1.027	0.7853	1.343
11	11:1129917	0.8479	1	0.9784	0.7824	1.223
11	11:573554	0.8495	1	0.9773	0.7708	1.239
11	11:378658	0.8509	1	0.9452	0.5251	1.701
11	11:1088976	0.8521	1	0.9254	0.4096	2.091
11	11:1675965	0.8526	1	0.9613	0.6339	1.458
11	11:328563	0.8528	1	1.033	0.7346	1.452
11	11:272010	0.8534	1	0.9798	0.789	1.217
11	11:1236427	0.8535	1	1.072	0.5121	2.245
11	11:1864346	0.8543	1	1.051	0.6179	1.788
5	5:150227966	0.855	1	0.941	0.4905	1.805
11	11:1802438	0.8564	1	1.021	0.8151	1.279
11	11:1413056	0.8571	1	0.9796	0.7828	1.226
22	22:18255988	0.8572	1	1.031	0.7419	1.432
11	11:2156168	0.8572	1	0.9258	0.4	2.143
20	20:36770588	0.8576	1	0.9737	0.7277	1.303
11	11:1122210	0.8584	1	0.9803	0.7883	1.219
11	11:1818095	0.8584	1	1.022	0.8062	1.295
11	11:1372934	0.8591	1	1.02	0.8213	1.266
11	11:370682	0.8593	1	1.024	0.7893	1.328
11	11:284538	0.8594	1	0.978	0.7644	1.251
11	11:619671	0.8603	1	1.027	0.7641	1.38
11	11:2072097	0.8624	1	1.031	0.7278	1.461

CHR	SNP	UNADJ	BONF	OR	L95	U95
6	6:36038758	0.8624	1	0.9154	0.3368	2.488
17	17:57992343	0.8625	1	0.9802	0.782	1.229
11	11:1589621	0.8654	1	1.049	0.6025	1.827
11	11:2143313	0.8656	1	1.056	0.5593	1.995
11	11:1490204	0.8685	1	1.03	0.724	1.466
11	11:1398316	0.8712	1	1.028	0.7392	1.429
11	11:297603	0.8714	1	0.9775	0.742	1.288
11	11:636496	0.8716	1	1.019	0.8144	1.274
11	11:237266	0.8729	1	1.071	0.4643	2.468
11	11:1290579	0.8732	1	0.9736	0.7005	1.353
9	9:21986847	0.8741	1	1.021	0.7912	1.317
11	11:1571747	0.8756	1	0.9333	0.3933	2.215
11	11:1975951	0.8757	1	1.031	0.7046	1.508
11	11:2021815	0.8767	1	1.018	0.8126	1.275
11	11:299517	0.8773	1	1.024	0.7544	1.391
11	11:1874404	0.8784	1	1.018	0.8089	1.282
11	11:1936085	0.8785	1	0.9483	0.4802	1.873
11	11:1314028	0.8789	1	0.9825	0.7827	1.233
11	11:1751702	0.88	1	1.055	0.5253	2.12
11	11:1834254	0.88	1	1.031	0.6925	1.535
11	11:702097	0.8809	1	1.017	0.8188	1.262
11	11:1684009	0.8814	1	0.9828	0.7819	1.235
11	11:1729593	0.8815	1	0.9823	0.7767	1.242
8	8:109796415	0.8819	1	1.045	0.5814	1.88
11	11:1377193	0.8826	1	1.044	0.5893	1.85
11	11:2113165	0.8842	1	0.9589	0.5454	1.686
11	11:1230925	0.885	1	0.9585	0.5398	1.702
11	11:1370414	0.8854	1	0.9593	0.5448	1.689
11	11:1683564	0.8856	1	0.9831	0.7797	1.24
11	11:1147708	0.8865	1	1.041	0.6015	1.801
6	6:36073831	0.8869	1	1.017	0.8017	1.291
11	11:715618	0.8898	1	0.949	0.4524	1.99
11	11:1673636	0.8902	1	0.9842	0.785	1.234
11	11:1624377	0.8914	1	0.9515	0.4663	1.941
11	11:1721982	0.8939	1	1.051	0.5079	2.173
11	11:1885225	0.894	1	0.9738	0.659	1.439
11	11:270330	0.8946	1	0.9719	0.6373	1.482
11	11:636433	0.8946	1	0.9773	0.6958	1.373
11	11:1972919	0.8951	1	0.985	0.7865	1.234
16	16:87430239	0.8956	1	1.016	0.8039	1.283
11	11:1856808	0.8957	1	1.037	0.6	1.793
11	11:1485492	0.8987	1	1.063	0.4146	2.726

CHR	SNP	UNADJ	BONF	OR	L95	U95
11	11:2108072	0.8988	1	0.985	0.7804	1.243
11	11:1010649	0.8996	1	1.025	0.6968	1.508
11	11:290494	0.8999	1	1.026	0.685	1.538
11	11:308180	0.9001	1	0.9853	0.782	1.241
11	11:1881256	0.9001	1	0.9833	0.7558	1.279
11	11:867621	0.903	1	1.032	0.6234	1.708
11	11:1854902	0.9041	1	1.054	0.4485	2.477
11	11:344351	0.9045	1	1.02	0.7403	1.405
11	11:2156213	0.9045	1	1.014	0.8104	1.268
11	11:1858262	0.905	1	1.023	0.7003	1.495
11	11:834597	0.9056	1	1.057	0.4212	2.654
20	20:36794497	0.9059	1	1.042	0.5287	2.053
11	11:299719	0.9061	1	1.014	0.8074	1.273
11	11:599779	0.9079	1	0.9831	0.7364	1.312
5	5:179260183	0.9113	1	1.049	0.4524	2.432
11	11:1657987	0.9137	1	1.023	0.6792	1.541
1	1:150714363	0.9142	1	1.032	0.5841	1.823
11	11:2003541	0.9145	1	1.012	0.8094	1.266
17	17:57976113	0.9147	1	1.039	0.5142	2.1
14	14:102552775	0.9158	1	1.013	0.8021	1.278
14	14:102568258	0.9166	1	0.9728	0.5807	1.63
6	6:31543758	0.917	1	1.021	0.6933	1.503
11	11:1659308	0.918	1	1.014	0.7803	1.317
11	11:1901165	0.918	1	0.9762	0.6176	1.543
11	11:599654	0.9184	1	1.024	0.6555	1.598
16	16:28502972	0.9194	1	1.021	0.6771	1.541
11	11:1600426	0.9199	1	1.02	0.6905	1.507
17	17:78087888	0.9206	1	0.9841	0.7179	1.349
11	11:270548	0.9213	1	1.02	0.6843	1.521
11	11:2074376	0.9264	1	1.013	0.7665	1.339
20	20:36787429	0.9268	1	0.9889	0.7799	1.254
11	11:795366	0.9271	1	0.9839	0.6944	1.394
11	11:1089507	0.9294	1	1.014	0.7443	1.382
11	11:1693217	0.9297	1	0.9898	0.788	1.243
6	6:31544189	0.9305	1	0.9854	0.7075	1.372
11	11:1644916	0.9307	1	1.011	0.7903	1.293
20	20:62703148	0.9309	1	0.9831	0.6693	1.444
11	11:846070	0.9318	1	0.979	0.6026	1.591
11	11:261906	0.933	1	0.9893	0.7706	1.27
11	11:370709	0.933	1	0.9895	0.7744	1.265
11	11:483855	0.9342	1	0.9714	0.4877	1.935
11	11:1620784	0.9343	1	1.01	0.8022	1.271

CHR	SNP	UNADJ	BONF	OR	L95	U95
11	11:2068717	0.9356	1	1.015	0.7144	1.441
13	13:113975768	0.9358	1	1.013	0.7443	1.378
11	11:1528777	0.9359	1	1.032	0.4754	2.242
22	22:18220831	0.9362	1	1.023	0.585	1.789
11	11:2049412	0.9364	1	1.02	0.6299	1.651
11	11:1391359	0.9372	1	1.009	0.7981	1.277
11	11:567391	0.9401	1	0.9905	0.7716	1.271
11	11:1417398	0.9405	1	1.018	0.636	1.63
11	11:1941395	0.9419	1	0.9814	0.5915	1.628
11	11:592700	0.9422	1	0.9664	0.3836	2.434
11	11:1366501	0.9437	1	0.9732	0.4579	2.069
11	11:1805061	0.9445	1	0.9837	0.6201	1.561
11	11:1665317	0.9446	1	0.9851	0.6441	1.507
11	11:2021075	0.946	1	0.9923	0.7936	1.241
11	11:968433	0.9468	1	0.9734	0.4412	2.148
17	17:66427519	0.9483	1	1.01	0.7438	1.372
11	11:378188	0.9492	1	0.9925	0.7865	1.252
11	11:1027811	0.95	1	0.9922	0.7776	1.266
11	11:2014709	0.9506	1	0.9929	0.7931	1.243
14	14:102561751	0.9525	1	0.9793	0.4921	1.949
11	11:229854	0.9533	1	0.9846	0.5869	1.652
6	6:36075219	0.9533	1	0.9925	0.7719	1.276
11	11:1729250	0.9543	1	0.9926	0.7704	1.279
11	11:343420	0.9559	1	0.9912	0.7247	1.356
11	11:308075	0.956	1	0.989	0.6681	1.464
11	11:1901467	0.9575	1	1.007	0.7802	1.3
1	1:150738197	0.9586	1	0.9929	0.7581	1.3
11	11:219967	0.9596	1	0.9798	0.4457	2.154
11	11:284094	0.9597	1	1.016	0.5473	1.886
11	11:1717922	0.961	1	1.01	0.6693	1.525
11	11:1075747	0.9612	1	1.006	0.8023	1.26
11	11:1653954	0.9639	1	1.006	0.7733	1.309
11	11:287120	0.9643	1	0.9918	0.6927	1.42
11	11:1439894	0.9662	1	0.9936	0.7377	1.338
11	11:1261561	0.967	1	0.9859	0.5033	1.931
11	11:2170143	0.9671	1	0.9952	0.7925	1.25
20	20:36791496	0.969	1	1.005	0.7844	1.287
11	11:480016	0.9705	1	1.01	0.5935	1.719
11	11:1920285	0.9728	1	1.004	0.7853	1.284
11	11:1652855	0.9734	1	0.9933	0.6709	1.471
11	11:1787698	0.9744	1	0.9872	0.4493	2.169
11	11:1698360	0.9759	1	1.003	0.803	1.254

CHR	SNP	UNADJ	BONF	OR	L95	U95
11	11:844862	0.976	1	0.9922	0.5938	1.658
11	11:1686790	0.9762	1	0.9946	0.6988	1.416
11	11:565872	0.9771	1	1.004	0.7817	1.289
11	11:1220760	0.9775	1	1.003	0.7905	1.274
11	11:515922	0.9795	1	0.9905	0.4767	2.058
11	11:353651	0.9805	1	1.005	0.673	1.501
11	11:873711	0.981	1	1.007	0.5891	1.72
11	11:408352	0.981	1	1.003	0.783	1.285
11	11:592222	0.9824	1	1.003	0.75	1.342
11	11:1231137	0.9827	1	0.9935	0.5518	1.789
13	13:48934934	0.9827	1	0.9945	0.605	1.635
11	11:1029135	0.9832	1	1.007	0.5117	1.983
11	11:2017464	0.9843	1	1.003	0.7427	1.355
11	11:1093898	0.9846	1	0.9945	0.5678	1.742
11	11:592666	0.9854	1	1.007	0.4862	2.085
11	11:1084362	0.9856	1	1.004	0.6778	1.486
11	11:816017	0.9865	1	1.003	0.7304	1.377
11	11:2154293	0.9891	1	1.005	0.4953	2.039
17	17:66429904	0.9894	1	1.002	0.7319	1.372
17	17:78089762	0.9905	1	0.9981	0.7299	1.365
11	11:2139662	0.9924	1	0.9983	0.7087	1.406
11	11:1375960	0.995	1	0.9993	0.8	1.248
11	11:1018711	0.9969	1	1.002	0.4096	2.45
11	11:589597	0.9972	1	1.001	0.7333	1.365
11	11:829254	0.9981	1	0.999	0.4199	2.376